TOWARDS THE DEVELOPMENT OF NOVEL ELISAS FOR AVIAN PNEUMOVIRUS (APV) SEROLOGY

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by

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Contents

Preface

The experimental work described in this thesis was conducted at the University of Liverpool in the Department of Veterinary Pathology, Jordan Building, Leahurst, UK between 2003 and 2006, during the tenure of a sponsorship awarded to the author by BioChek, Craberth straat 38-C, Gouda, Netherlands.

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Abstract

The thesis describes experimental work undertaken to develop novel avian pneumovirus (APV) ELISAs using subtype A recombinant viral proteins as antigen. It also investigates regions within the fusion protein (F) that may be important in the protective immune response to the virus.

Some potentially antigenic APV proteins, namely the F protein, the nucleocapsid protein (N) and the phosphoprotein (P) were selected for expression in *E.coli* and baculovirus systems. In addition to APV proteins the green fluorescent protein (GFP) was used as a direct visual reporter of expression. Initially an in house expression vector (p18smahis) was developed for use in *E.coli* and although cloning of selected genes into this vector was successful overall expression of them was low. Expression of these proteins and new GFP-APV fusion proteins was significantly improved when a commercial *E.coli* expression vector p-ET30 was used in replacement for p18smahis. Purification of these proteins was through Ni_2+ capture of designed 6 x his tag regions placed at either or both the N and C terminal of each protein. Only GFP was successfully purified as native protein although, purification of native N and P was successful after the proteins had been linearised using 8M urea. This highlighted problems with concealment of his tag regions within the proteins themselves which rendered them inaccessible for capture. In addition, GFP-APV fusion proteins indicated problems with toxicity as *E.coli* cells expressing them appeared to lose structural integrity as early as 1 hour post induction. As a result six regions of the F gene were individually cloned into the same expression vector. Hydrophobic regions were excluded in the design of these new sections in the attempt to eliminate toxic effects and reduce size of proteins to minimize concealment issues. Each new F section was expressed as a his tagged recombinant protein. These were detected using an anti his tag monoclonal antibody. Five of the six F sections consistently produced higher absorbance readings with subtype A, B and C APV-positive antisera and lower readings with specific pathogen free (SPF) serum from 12 week old chickens, when compared with a Liverpool-developed whole virus antigen ELISA. Tests suggest that subtype A F antigens 1 – 5 (amino acids 25-70, 77-96, 146-199, 211-310 and 336-479 respectively) and in particular 4 F protein are strong universal antigen candidates for APV ELISAs.

In addition to their use in ELISAs, a study of F protein fragments enabled the identification of two regions targeted by neutralizing antibodies, one of which is also within an equivalent region identified for respiratory syncytial virus (RSV) F protein, suggesting that the equivalent regions of the F proteins in the two related viruses share similar functions.

A baculovirus expression system was also used to express a region of the F protein and GFP. In accordance with *E.coli* expressed proteins these were 6 x his tagged for purification. Generation of plaque purified F2 and GFP recombinant baculovirus was successful and a good level of expression was observed, as was best demonstrated by GFP using ultraviolet (UV) microscopy. However, F2 protein could only be purified from insoluble material. Unfortunately this made it unsuitable as an ELISA antigen due to destruction of its conformational epitopes.

List of presentations

Naylor, C.J., Brown, P. A., Edworthy, N., Ling, R., Jones, R. C., Savage, C. E., Easton, A. J., *Development of a reverse-genetics system for Avian pneumovirus demonstrates that the small hydrophobic (SH) and attachment (G) genes are not essential for virus viability.* J Gen Virol, 2004. 85(Pt 11): p. 3219-27.

Brown, P. A., Ricchizzi, E., Bonci, M., Jones, R. C., Naylor, C. J *Towards the development of novel ELISAs for avian pneumovirus serology.* Paper presented at the V. international symposium on avian corona- and pneumoviruses and complicating pathogens, Rauischholzhausen, Germany 14-16 May 2006.

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Acronyms and abbreviations used

aa	amino acid
ABC	advanced biotechnology centre
AcMNPV	*Autographa californica* multiple nuclear polyhedrosis virus
AGP	agar gel precipitation
APV	avian pneumovirus
BCA	bicinchoninic acid
bis	bisacrylamide
bp	base pairs
bRSV	bovine respiratory syncytial virus
BSA	bovine serum albumin
^0C	degree Celsius
CBC	carbonate-bicarbonate buffer
CD_{50}	50% ciliostatic dose
CEF	chick embryo fibroblasts
CELi	chick embryo liver
cm	centimetre
CO_2	carbon dioxide
COOH	carboxy terminal
CPE	cytopathic effect
DAB	diamino benzidine
DNA	deoxyribonucleic acid
ELISA	enzyme-linked immunosorbent assay
EM	electron microscopy
F	fusion protein
F0	full length F protein

G	attachment protein
g	gram
GAT	goat anti turkey
GFP	green fluorescent protein
GOI	genes of interest
HMPV	human metapneumovirus
HRP	horse radish peroxidase
hrs	hours
hRSV	human respiratory syncytial virus
IF	immunofluorescence
IFN	interferon
IIF	indirect immunofluorescence
ILTV	infectious laryngotracheitis virus
IP	immunoperoxidase
IPTG	Isopropyl β-D-thiogalactopyranoside
ISGs	IFN-stimulated genes
Kb	kilobases
kDa	kilodalton
L	polymerase protein
2ME	2-Mercaptoethanol
M	matrix protein
mA	milliamps
MAbs	monoclonal antibodies
µg/ml	microgram/millilitre
mg	milligram
Mg	*Mycoplasma gallisepticum*
mins	minutes
µl	microlitre

ml	millilitre
mm	millimetre
mM	milli-Molar concentration
M_r	relative molecular mass
mRNA	messenger ribonucleic acid
MW	molecular weight
N	nucleocapsid protein
N	amino terminal
NC	nitrocellulose
NDV	newcastle disease virus
nm	nanometre
nt	nucleotides
OD	optical density
OPD	o-Phenylenediamine
P	phosphoprotein
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pi	post infection
RBS	ribosomal binding site
RE	restriction endonuclease
Rf	relative frontal mobility
RNA	ribonucleic acid
RNAi	RNA interference
rp	repressor protein
rpm	revolutions per minute
RSV	respiratory syncytial virus
RT-PCR	reverse transcriptase polymerase chain reaction
SDM	site-directed mutagenesis
SDS	sodium dodecyl sulphate

SH	small hydrophobic protein
SHS	swollen head syndrome
siRNA	short interfering RNA
SN	serum neutralization
SPF	specific pathogen free
T7	T7 polymerase
Taq	thermus aquaticus
Tg	target gene
TOC	tracheal organ culture
TRT	turkey rhinotracheitis
TRTV	turkey rhinotracheitis virus
U	uracil
UV	ultraviolet
VN	virus neutralization
w/v	weight/volume
wk	week

Chapter 1

Introduction and aims

Contents **Page**

Chapter 1

Introduction and aims

1.1 Introduction

Diagnostic techniques used to identify the causal agents of economically important virus diseases of domestic poultry need to be rapid, sensitive and specific so that appropriate methods of control can be implemented. There is a wide range of tests available to detect viral antigens such as immunofluorescent staining, virus isolation and the polymerase chain reaction (PCR).

For detection of specific antibodies in serum enzyme-linked immunosorbent assays (ELISAs) are the method of choice, since these tests are rapid, specific, require only small volumes of serum and are able to process many samples at the same time. Most are also commercially available. The sensitivity and specificity of these tests are continuously under scrutiny. Traditionally most ELISAs use antigens prepared from virus-infected cell cultures and these generally work well, but have some disadvantages. These assays require virus that can easily be cultured in vitro and this can sometimes make it difficult to respond quickly to the emergence of new field strains. Furthermore, preparation methods of the whole virus often contain unwanted cellular proteins, which can lead to non-specific binding, which in turn reduces the specificity of the test.

Novel ELISAs have been developed to combat this, and one approach has been to use recombinant proteins [1-8] This enables the expression of individual epitopes that may be group or subtype-specific. Each of these authors reported increased or equal sensitivity and specificity of recombinant ELISAs with conventional ELISAs. Furthermore Chen et al. [8] highlighted some important

advantages of using recombinant proteins over whole virus-infected cell extracts as antigen. These include safety (being non infectious) and having the potential to be modified rapidly in respond to new viral strains

The present study has been concentrated around avian pneumovirus (APV), initially named turkey rhinotracheitis virus (TRTV). Turkey rhinotracheitis was first described in South Africa in 1978 [9] and was later detected in Europe [10]. Here, the virus was isolated and identified as the primary agent [11-13] then characterized as a pneumovirus [14-16]. APV is now known to exist in four subtypes A, B, C and D. This virus has been the major respiratory pathogen of turkeys in several countries for the last 20 years. Although its role as a primary pathogen in chickens is not clearly defined, it is known to be involved in respiratory disease. [17-19]. The initial viral infection is often complicated by secondary bacterial infections, which results in high morbidity and variable mortality [20]. In turkey hens, APV infection can induce reductions in egg production, and infection of birds of any age can result in serious economic losses [21, 22].

Initially the detection of APV was through immunofluorescent staining of infected material and isolation techniques, of which the most commonly used was tracheal organ culture (TOC). However, advances in molecular techniques have allowed faster detection of viral particles through the development of reverse transcriptase polymerase chain reactions (RT-PCR). Although RT-PCRs allow rapid detection they require careful handling and expertise as their high sensitivity means that contamination with extraneous DNA is a consistent threat. In some cases it is necessary to use all the available detection methods.

Virus neutralization and indirect fluorescent assays were the initial methods used for detecting APV antibodies, however progress in culturing APV lead to developments in ELISA assays with more efficient and rapid detections of antibodies. These assays are now the method of choice for antibody detection although for reasons outlined above improvements are required.

1.2 Aim

It has been shown that ELISAs which use an empirical sub-type A antigen do not detect antibodies to sub-type B with the same sensitivity, and vice versa [23]. Thus the use of a heterologous antigen in an ELISA may lead to false negatives, which, in turn could be interpreted as apparent vaccine failure or as absence of APV infection. By using individual APV viral proteins as antigen it may be possible to detect antibodies to epitopes that are equally sensitive across subtypes or be subtype-specific. Our approach has been to attempt to express important viral genes of subtype A APV encoding the fusion protein (F), the nucleocapsid protein (N) and the phosphoprotein (P), in both prokaryotic and eukaryotic systems, namely *E.coli* and insect cells respectively. These recombinant proteins have been purified using nickel column purification. Purified proteins were tested both for suitability as candidate antigens against a range of APV antisera and to investigate regions within individual proteins important in the protective immune response.

In addition to APV genes, a gene encoding the green fluorescent protein (GFP) of the pacific jellyfish *Aequorea victoria* was expressed. This protein was invaluable in both prokaryotic and eukaryotic systems when evaluating the steps throughout

expression and purification, as its inherent fluorescence allowed simple illumination with UV light without the requirement of additional factors.

Chapter 2

Literature review

Contents **Page**

Chapter 2

Literature review

2.1 Introduction

Avian pneumovirus (APV) initially named turkey rhinotracheitis virus (TRT) is recognised as a major respiratory pathogen of turkeys and chickens around the world. In chickens, the role of the virus as a primary pathogen is less clearly defined as in the turkey, although it is commonly, but not always associated with swollen head syndrome (SHS) in broiler breeders [24]. The disease was first described in turkeys in South Africa in 1978 [9] and was later detected in Western Europe [10]. Here, the virus was isolated and identified as the primary agent [11-13] and subsequently characterized as a pneumovirus [14-16]. It has now been classified in the new genus *Metapneumovirus* based on the absence of two genes found in pneumoviruses proper that code for non-structural proteins NS1 and NS2 [25, 26]. The avian viruses were the only members of this new genus until studies in Holland showed that metapnumoviruses exist in humans and had been present for at least 50 years [27].

There are different sub-types of APV. A and B are present in Western Europe and most other countries around the world with sub-type C only present in the USA and to a limited degree in France. Furthermore there has been a recent report from France of an APV strain isolated from turkeys that is genetically different from A, B and C, this is referred to as subtype D [28]. Varying degrees of nucleotide and amino acid (aa) sequence heterology exist between these subtypes and these can be used for differentiation. Serological differences not seen between subtypes A and B have now been demonstrated for subtype C

through the use of monospecific antisera and monoclonal antibodies (MAbs) in neutralization tests. It has therefore been suggested that this virus could represent the first isolate of a second serotype of APV. [29].

There have been reports of APV infection in species other than the turkey and the chicken some of which have been incriminated in the spread of infection such as wild or feral birds [30] [31]. However conflicting reports about APV infection of wild birds could suggest that they are susceptible to APV infection but may not play a role in transmission of infection. This notion is supported by the apparent absence of APV in Australia [32] which receives migratory birds from countries infected with APV in South East Asia and by the absence of subtype C in Central and South America although it is present in North America which shares migratory routes. The reverse applies when considering the absence of subtype A and B in North America.

2.2 The Virus

2.2.1 Taxonomy

Based on the morphology of viral particles, polypeptide analysis, RNA sequencing and mRNA analysis, APV has been placed in the family *Paramyoxoviridae*, genus *Metapneumovirus* in which it is now the type species [13-16, 25, 26, 33]. APV was initially grouped in the genus *Pneumovirus* because of its close genetic homology with human and bovine respiratory syncytial viruses (hRSV and bRSV respectively) as well as other mammalian pneumoviruses. However it has now been moved to the new genus *Metapneumovirus* together with human metapneumovirus (HMPV) based on the

differences in gene order after the M gene and on the absence of two genes that code for the non-structural proteins NS1 and NS2 [25, 26] (Figure 1)

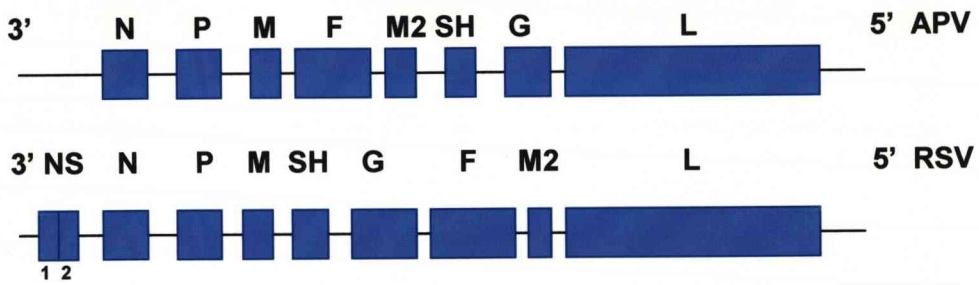


Figure 1. Comparison of genes and gene order between *Metapneumovirus* APV and *Pneumovirus* RSV both of which belong to the family *Pneumoviridae*.

2.2.2 Morphology

Studies of APV by negative staining electron microscopy (EM) show the virus in pleomorphic, often bizarre shapes that are enveloped and covered with a fringe of projections approximately 15 nanometre (nm) in length (Figure 2). The virus particles have a size range of 50nm to more than 200nm in diameter and although these are often spherical they can exist in long filamentous forms of over 1000nm in length. The helical nucleocapsid which contains the viral genome is 14nm in diameter with a 7nm pitch [10, 11, 13, 14, 33-36].

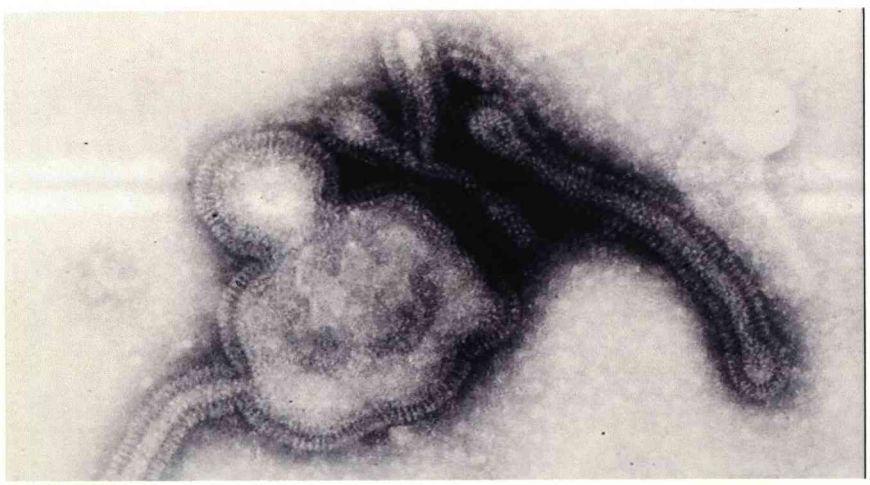


Figure 2. Electron micrograph of negatively stained APV, showing its pleomorphic nature and surface projections (R. C. Jones)

2.2.3 Physiochemical properties

APV has a buoyant density of 1.21g/ ml in sucrose gradients [14]. It is stable between pH 3.0 to 9.0, inactivated at 56^0C after 30minutes and does not possess heamaggluting properties [13, 35].

2.2.4 Nucleic acids

APV has a single-stranded negative-sense RNA genome [37]. Sub-type A genes have all been sequenced [38-45] giving a final genome length of 13,373 nucleotides (nt) [42]. All of the genes of sub-type B have now been sequenced [38, 41, 46-48] except for the L gene (currently being addressed at Liverpool), therefore the total length has not yet been reported. All genes of subtype C have also been sequenced [49-56], however there is some debate over its total length [57, 58]. This is a result of discrepancies in the length of the G gene and the presence or absence of the SH gene [46, 50, 53, 54, 59]. Only the G gene of sub-type D has been sequenced in its entirety with partial sequencing of the F and L genes [28]. Lwamba *et al..,* 2005 state that European viruses share 97-99% nt sequence identities within a subtype but only 56-61 % across subtypes. Isolates from the USA demonstrated nt identities between 62 and 65% with subtype A, 60-63% with subtype B and 89-94% within a subtype [60]. This type of data has not yet been reported for subtype D. However the full G gene shares 56.6% nt identity with subtypes A and B, the partial F gene shares 73.7, 80.5 and 70% nt identity with A, B and C respectively and the partial L gene shares between 75.8 and 76.1 % nt identity with subtype A [28].

2.2.5 Polypeptides

The genome of APV is transcribed into eight mRNAs encoding eight proteins. The genome order (designated according to the proteins) is as shown in Figure 3. These proteins are organized into the packaged virus as shown in diagrammatic form in Figure 4.

Before it was appreciated that different sub-types existed, three groups reported the sizes of the individual APV polypeptides. Sizes ranged from 14-200 kilodalton (kDa) [14-16, 61] and later studies confirmed these isolates to be of an A subtype [47]. These were summarised by Cavanagh and Barrett [16] giving the following; L: 200kDa, G: 82-84kDa, F0: 68kDa dissociating into F1: 53-54 kDa and F2: 14-15kDa (in reducing conditions), N: 38-43kDa, P: 35-40kDa, M: 30-35kDa and 19-22kDa unidentified. The G and F proteins were found to be glycosylated. Differences in the nt length of each gene between subtypes A and B are minimal, with C only differing substantially in its G gene. Therefore, the molecular masses of each polypeptide should remain faithful to the published sizes with the exception of protein G of subtype C.

Three proteins distinguish APV from the closely related family members morbilliviruses and paramyxoviruses which initially grouped it with other pneumoviruses such as RSV. However, the absence of two non-structural proteins N1 and N2 found in other pneumoviruses is the basis for classification of the new genus *Metapneumovirus* [25, 26]. The N and P proteins of pneumoviruses are smaller than those found in morbilliviruses or paramyxoviruses and the small polypeptide of 22kDa termed the M2 protein [62] is not found in either of those genera.

The SH and G proteins of subtypes A and B share 47 % and 38% aa identity respectively [46, 47] and aa identities for N, P, F, M and M2 range between 83-91% [38, 41, 48, 60]. Identities between the L proteins of A and B are unavailable. Subtype C proteins; N, P, F, M and M2 have 53%-78% aa identities with those of subtype A viruses and 52%-77% with those of subtype B viruses [51, 52, 60]. The G and SH proteins of subtype C share only 14 and 19.5% aa identity with subtype A respectively and only 14 and 17.7% respectively with subtype B [50, 63]. The G protein of subtype D shares 29.5% aa identity with subtype A and 31.2% with subtype B and 16-21% with subtype C. Partial sequencing and deduced aa sequence of the F protein when compared with homologous regions in subtypes A, B and C gave aa identities of 89.7, 97.2 and 77.6% respectively. Partial L aa sequences yielded identities between 85.3 and 84.3% with subtype A [28, 50].

Fig 3.APV genome order, designated according to the proteins. N (nucleocapsid protein) P (phosphoprotein) L (polymerase protein) are the nucleocapsid proteins. M2 appears to play a regulatory role in RNA synthesis. M is the matrix protein. SH (small hydrophobic protein) G (attachment protein) and F (fusion protein) are the transmembrane glycoproteins.

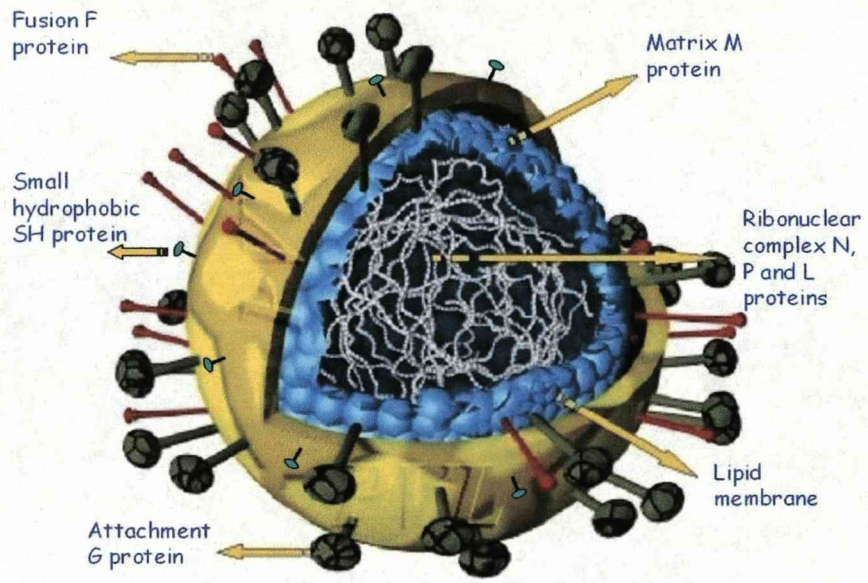


Figure 4. Diagrammatic representation of a pneumovirus kindly provided by Andrew Easton University of Warwick

2.2.6 Replication

Very little is known about the mechanisms of: (i) cell attachment, (ii) penetration, (iii) transcription, (iv) replication and (v) assembly and release of APV. However, there are some published models for pneumoviruses in general.

(i) & (ii) Attachment and penetration

Conventional models for attachment suggest that G proteins interact with glycosaminoglycans or heparin-like molecules [64-68] on the target cell surface [69]. It is proposed that after attachment, the F glycoprotein promotes a pH-independent fusion between the virus envelope and the target cell membrane [70]. This introduces the individual viral components to the cytoplasm of the cell where replication can take place. Some questions have been raised about the conventional model for attachment, suggesting that the process may be more

complex. Heminway *et al.* [71] suggested that the hRSV fusion event required all the surface proteins F, G and SH. However, recent reverse genetic approaches for pneumoviruses have produced several viral mutants that lack either the SH, G or both which are still able to infect cells *in vitro*. Naylor *et al.* [72] in developing such a system for APV, produced a virus that lacked both the SH gene and the G gene. This virus grew more slowly than its wild type progenitor and produced atypical cytopathic effect (CPE) with much larger syncytia. This work confirms that the SH and G proteins are not essential for virus viability but are essential for normal growth characteristics *in vitro*. Studies on RSV have shown similar results [73-75]. These findings imply that like RSV, APV has an auxiliary attachment function, probably linked with the F protein. It has been suggested that the enveloped glycoproteins also play a role in assembly through their potential to bind viral components within the host cell [76].

(iii) Transcription

The transcription and replication models of pneumovirus genomes are consistent with those of all nonsegmented negative-sense RNA viruses.

Transcription begins at a single polymerase entry site (termed the leader sequence) that is located at the 3' end of the genome. The polymerase complex now moves along the gene until it reaches a region that marks a point for termination. During the transcriptional process the mRNA is methylated, producing a capped structure that prevents degradation and ensures translation on host cell ribosomes. Termination signals of pneumoviruses consist of a small run of uracil (U) residues, these also mark the position where the polyadenylated tail is added. Once these processes have been completed the mature mRNA is

released for translation on free or membrane bound ribosomes. It has been suggested that only about 50% of the polymerase molecules are able to continue moving along the genome to transcribe the next gene [70]. The remaining, dissociated polymerases, translocate to the 3' terminal leader sequence to begin transcription at the first gene. This dissociation versus continuation process continues down the genome. Thus genes that are closer to the 3' leader sequence are more abundant as mRNA molecules than those closer to the 5' end. However, it has been shown that all eight RSV genes direct the production of more read through mRNAs in the presence of the M2-1 protein [77]. Krempl *et al.* [78] demonstrated the increase of expression of genes closer to the 3' leader sequence, by shifting the G and F proteins of hRSV to its promoter-proximal positions. In doing this these authors increased the mRNA expression by 2.4 and 7.8 fold respectively. Protein expression was increased by approximately 2.5 fold.

(iv) Replication

The model of replication of pneumoviruses is similar to that of other non-segmented negative sense RNA viruses and it is proposed that the nucleocapsid complex is the functional unit and that the N, P and L proteins are involved. Replication begins with the polymerase protein binding to a replication promoter sequence at the 3' end of the genome. These regions are similar but not identical to the transcription promoters with which they overlap [79]. Unlike transcription, the polymerase is now committed to continue to the end of the genome generating an antigenome template. This template is then used for continued copying. The presence of increased N expression has been shown to stimulate replication of hRSV suggesting that replication is dependant on RNA

encapsidation [80]. However, Bermingham et al. [81] have also shown that the M2-2 protein of the same virus plays a regulatory role in the balance between replication and transcription.

(v) Assembly and release

The M protein is assumed to play a major role in virus assembly as are the enveloped glycoproteins. It is thought that a series of interactions between the cytoplasmic domains of the enveloped glycoproteins and M protein, which acts as a bridge to the neucleocapsid complex, results in progeny virons budding from the cell surface. Roles of the glycoproteins in virus assembly have been established for several members of the paramyxoviridae family such as: (i) the fusion protein of Newcastle disease virus (NDV) [82] (ii) the heamagglutinin-neuraminidase protein of Sendai virus [83] (iii) the haemagglutanin and neuraminidase proteins of influenza virus [84] (iv)the G protein of vesicular stomatitis virus [85], (v) the E2 protein of Semliki Forest and Sindbis virus [86] and (vi) the G protein of RSV [76]

The M protein of RSV has also been shown to interact with the membranes of infected cells as well as with various viral components [87, 88]

2.2.7 Antigenicity

Early studies on three UK isolates and one French isolate of APV using double immuno-diffusion, indirect immunofluorescence (IIF), serum neutralization tests (SN) and ELISA using polyclonal sera, showed that all four were antigenically similar [34, 89]. Later studies using monoclonal MAbs [90, 91] indicated that isolates collected between 1985 and 1990 from the UK, were themselves closely

related and that they were very similar to a strain isolated in South Africa in 1978. Conversely, the UK isolates were shown to be somewhat different from isolates from France, Hungary, Italy, Spain and Holland [91]. In agreement with this, was the work of Collins et al. [90], they found that the French Isolate 1556 [10] was very similar to isolates made in the UK prior to 1990. These data showed that the different isolates were clearly related but could be separated into two distinct groups. Juhasz and Easton [47], in a landmark paper, confirmed this by demonstrating extensive sequence variation in the attachment G proteins of these isolates and subsequently named the two groups subtypes A and B. In that study, isolates of subtype A comprised UK and French strains and isolates of subtype B were formed from the other continental European strains.

Isolates of APV from the USA have demonstrated antigenic differences from both A and B subtypes and have subsequently been designated as subtype C. Preliminary neutralization tests using mono-specific polyclonal antisera to A or B demonstrated a failure of these sera to neutralize the first US isolate from Colorado and *vice versa*. Likewise MAbs that neutralized A and B also failed to neutralize C [29]. However, Cook et al. [29] did show a partial neutralization of subtype C with subtype A hyperimmune serum.

The differences seen antigenically can be explained by the low aa identities of subtype C proteins with subtypes A and B proteins, especially in the highly antigenic surface proteins (see section on polypeptides). The use of recombinant proteins is helping to identify regions of antigenic cross-reactivity [92].

2.3 Disease

2.3.1 Hosts

The natural host of APV is the turkey and to some degree the chicken although the role of the virus as a primary pathogen in the latter species is less clearly defined. Evidence of infection with subtypes A and B has been reported in a number of other species including ducks [93]. Susceptibility to APV infection has been demonstrated serologically in pheasants by ELISA and virus neutralization (VN) [94, 95], in guinea fowl by ELISA and VN [95, 96], in ostriches from Zimbabwe by ELISA [97] and in herring gulls from the Baltic Coast, Germany by VN [98]. Subtype C APV in the USA has been detected in wild Canada geese and the blue-winged teal [99] house sparrows, ring-billed gulls and snow geese [100] Turpin [101] Detected subtype C antibodies to APV in five of fifteen different species of wild bird.

2.3.2 Geographical distribution

APV has now been detected globally with few exceptions. The only large land mass where it remains undetected is Australia [32] and there are no reports from Canada. It was first detected in South Africa in turkeys [9] and subsequently in the UK [102]. Other countries include, France [10], Spain [102], Italy [102], Israel [103], Germany [104] Hungary [105], Taiwan [106] Japan [107], South and Central America [19, 108] the Dominican Republic [109] and North America [110]. Subtype distribution is as follows. It appears infections in the UK, Europe, Africa, Central and South America and Asia have been of the A and/or B subtypes with C subtype infections, until recently, have been found only in the USA. However, a Colorado-like APV virus was isolated from ducks in France [93]. This was confirmed as a European C strain of APV but has been shown to

have a different genetic lineage [111]. Two more French isolates Fr/85/1 and Fr/85/2 were shown to differ from A, B and C through sequence analysis of their F, G and L genes and thus a fourth subtype, labelled D, has been identified here [28] [111].

2.3.3 Clinical disease

In general, the severity of the disease is greater in the field than under experimental conditions [21]. Typically, in experimental conditions in turkeys, the characteristic clinical signs of APV infection begin with clear nasal discharge, which later becomes mucopurulent. 5 days post infection (pi) there is an increase in ocular discharge which frequently becomes frothy. During this period there are also signs of depression, head shaking, coughing, sneezing and a swelling of the infraorbital sinuses [112]. Within 10 – 12 days full recovery maybe observed [11, 13, 35, 36]. Importantly Naylor, Jones and Williams observed that in isolation conditions the housing of turkeys even on clean litter as apposed to wire resulted in more consistent and severe clinical signs (unpublished observations).

Naylor and Jones 1993 [112] reported that the severity of clinical sings varies depending on environmental and management factors such as ventilation, hygiene and stocking densities. In the field, other adverse effects are seen. There is poor feed conversion in growing birds, and in layers there is a drop in egg production down to 40% of the expected levels and in worst cases as low as 2% [112]. Recovery has been shown to take approximately three weeks [21, 102]. Mortality is variable but in cases of poor management conditions and the resulting invasion of secondary agents, it has been shown to reach up to 30% or even over 50% [21, 102]. The role of secondary agents in disease severity has

been demonstrated in experimental conditions. *Bordetella avium, Pasteurella*-like organisms [113] and *Mycoplasma gallisepticum* (Mg) [114] (all of which are commonly associated with secondary infections in the field) have been shown to exacerbate and prolong the clinical disease. It has been suggested that the failure to develop severe clinical disease in experimental conditions is due, in part, to insufficient numbers of these secondary pathogens at critical times and the different management conditions [112].

Experimental infection of chickens with APV has produced milder clinical signs than in turkeys [115-117]. However, SHS is a pathological condition that affects all types of chickens and sometimes follows APV infection in broiler-type birds. Morley and Thomson [118] reported that broilers were affected at 4 to 6 weeks of age while Pattison et al. 1989 and O'brien 1985 [24, 119] reported that broiler breeder birds were affected from peak of lay, at about 30 weeks to 52 weeks. Clinical signs include depression, lack of locomotion, sneezing, coughing, head shaking and scratching. The most striking feature is subcutaneous oedema around the eyes [24, 118, 119]. Often this extends over the whole area of the head giving the face a swollen appearance, hence the name. Ear and eye discharge, red conjunctivitis, foul smelling green diarrhoea and severe tracheitis due to secondary *E.coli* pathogens have also been observed [118, 119].

SHS has never been reported following experimental infection with APV alone.

2.3.4 Effects of age and breed

Turkeys of all ages are susceptible to APV however the severity of the disease is more pronounced in younger poults [21, 120]. In experimental conditions

Williams *et al.* 1991 [121] had to increase the effective dose of 4.7 log10 for birds of 12 weeks to 5.2 log10 for birds of 22 weeks of age to induce similar clinical signs. There is no evidence to link differences in breeds with differences in APV susceptibility.

2.3.5 Pathogenesis

(i) Replication and persistence in the tissues

APV predominantly infects the upper respiratory tract of turkeys and chickens. Experimental infections by eyedrop or intranasal inoculation, have shown the virus replicating in epithelial cells of the nasal turbinates, trachea and sometimes lungs [35, 113, 116, 122-124]. Viral particles have been demonstrated in the trachea from days 1 – 7 post experimental infection (pi) and in the turbinates from days 2 – 6 using immunofluorescence staining (IF) or immunoperoxidase (IP) staining [122, 123]. Viral replication has also been demonstrated in the ciliated epithelial cells of the bronchus on days 4 and 5 pi [123].

In mature turkey hens, the virus has been detected in the reproductive tract after infection via the respiratory route. Jones *et al.* [122] detected viral replication in the epithelium of the uterus on day 7 pi and in all regions of the oviduct on day 9pi using IF staining. However, virus was only isolated from the middle magnum and vagina on day 9 pi. O'loan and Allan, [124] also detected virus in the surface epithelial cells of the uterus using IP staining.

The increasing use of RT- PCR as a diagnostic tool has also lead to improved sensitivity for virus detection. As a result, detection of virus from 17 to 19 days pi

[125] and as late as 28 pi [126] have been recorded in turkeys and chickens respectively under experimental conditions. However, isolation of virus is usually possible only between days 2 and 7 pi. Whether there is a low level of virus replication during these later stages, or indeed if the birds are still harboring infectious virus which becomes reactivated is unknown.

Dual infection of turkeys with bacterial pathogens commonly associated with field disease, lead to increased invasiveness of APV with virus being recovered from heart, liver, spleen, kidney and caecal tonsil [113]. Conversely, dual infection of APV and Mg did not increase the invasiveness of APV but did increase the invasiveness of Mg [114]. A recent paper on dual infection with vaccine strains of APV and NDV using one-day old specific pathogen free (SPF) White Leghorn chicks, showed APV detection, via RT-PCR for almost twice as long in dual vaccinates than in single vaccinates [127]. These authors suggest that after simultaneous vaccination of chicks with live APV and NDV vaccines there is a temporary suppression of APV vaccine proliferation.

In SPF chicks the virus has been isolated for 6 days pi from nasal secretions after experimental infection at one day of age with a turkey strain (BUT# 8544) [117]. Jones *et al.* [128] have also reported isolation of virus from the trachea on day 6 pi in chickens infected with a chicken strain. Cook *et al.* [116] demonstrated some different biological properties between two APV isolates, one of which was from turkeys and the other from chickens. These authors reported that both viruses principally replicated in the upper respiratory tact. However, the chicken isolate replicated to very high titre in the nasal tissue of both species, approximately $\log_{10}$ 6.0 median ciliostatic doses of virus per g (CD_{50}), whilst the turkey isolate only reached the same level in its host species. It is worth

emphasizing that chickens and turkeys can each be infected with viruses from either species.

The demonstration of virus in the reproductive tract of chickens has only been possible after experimental inoculation, via the unnatural, intravenous route [129]. These authors refer to a paper by Catelli *et al.* [130] and suggest that their failure to detect virus in the reproductive tract might be explained by the difference in age of birds used in the experiments, and relating it to the immaturity of the oviduct. However, it is important to note that Catelli *et al.* [130] inoculated birds via the oculonasal route only.

(ii) Macroscopic lesions

Experimental infection of day old poults with a virulent or non virulent strain of APV did not result in any abnormalities on postmortem examination. However, thickened air sacs were observed in poults infected with the virulent strain of APV together with bacteria. This thickening of the air sacs became severe between days 7 and 14 pi [113].

Following experimental infection of 30 week old laying turkeys with APV alone, Jones *et al.* [122] initially found clear, watery exudate in the turbinates, which later became mucoid and greyish in colour between days 1 to 9 pi. Excess mucus was found in the trachea between days 1 to 7 pi. The same paper reports several reproductive tract abnormalities in mature female turkeys including ovary regression, folded shell membrane in the oviduct, egg peritonitis, misshapen eggs and deposits of solid yolk material scattered throughout the abdomen. The

most common finding was the presence of whitish masses of inspissated albumin scattered throughout the organ.

Following natural APV infection together with secondary bacterial infection in the field, pericarditis, perihepatitis, air-sacculitis, pneumonia and adhesions between the pericardial sac and the epicardium have been described [21, 102].

In APV infected chickens, the only significant lesions noted are those associated with SHS in broilers or broiler breeders. These include extensive yellow gelatinous to purulent odema in the subcutaneous tissue of the head, wattles and neck. Swelling of the infraorbital sinuses has also been seen [104, 106, 107, 131]. Simmilar lesions have been seen in commercially raised pheasant poults in Japan [132].

(iii) Histopathology

Histopathological changes have been observed in the trachea [36, 96, 123], turbinates [96, 123] and less frequently the lungs [123]. After experimental infection of seven week old turkeys, Jones et al. [36] were able to demonstrate a loss of cilia and extrusion of the epithelial cells from the mucosal surface in the trachea by day 4 pi. These authors also showed an infiltration of inflammatory cells in the mucosa and submucosa of the trachea by day 4 pi.

Workers in France [96] repoted histopathological changes in the larynx, nasal cavities and trachea of both chickens and turkeys that had been experimentally infected at 4 weeks of age. However, changes were of a lesser degree in the trachea. They also reported acidophilic cytoplasmic inclusions in the ciliated

cells of the turbinates and the trachea, a finding that has not been reported by any other workers.

Majo *et al.* [123] observed changes in the mucosa and submucosa of the bronchi subsequent to experimental infection of chickens and turkeys. These consisted of inflammatory exudate in the brochial lumen, an increase in glandular activity, hyperplasia of the bronchial epithelium and the presence of an abundant mononuclear inflammatory infiltrate in the submucosa of the bronchi.

Following experimental infection of 3-week-old commercial broiler chickens with a turkey isolate, Jones *et al.* [117] demonstrated either focal heterophil leucocyte infiltration of the congested nasal epithelium or focal karyorrhexis of the tracheal epithelium in a small number of birds at day 4 pi. In uncomplicated APV infections the respiratory tissues usually return to normal after about ten days.

2.3.6 Immunity

(i) Humoral immune responses

ELISA, SN tests and IIF methods have been used to detect humoral antibody responses of turkeys or chickens to APV [133-140]. Following natural infection of APV in turkeys, Baxter-Jones *et al.* [134] in using three antibody detection methods demonstrated VN antibodies as early as 5 days after the initial appearance of clinical signs. These antibodies reached peak levels at this time as did IIF antibodies. However neutralising antibodies were decreasing by day 13. Conversely, ELISA antibodies had reached their peak on day 13. Antibodies were detected by all methods up to 34 days although serum titres were generally decreasing. Jones *et al.* [122] using experimental APV infection

of laying turkeys demonstrated ELISA and neutralising antibodies at high levels throughout their observation period of 89 days. Again in this study neutralising antibodies were detected before ELISA antibodies.

The inoculation of poults with attenuated strains of APV has also resulted in seroconversion and protection against virulent challenge [121, 141]. Despite the humoral response following APV infection, the role of these antibodies in protection of the respiratory tact is unclear. Jones *et al.* [142] produced evidence that would suggest that the cellular response provides the main resistance to the virus in the upper respiratory tract. These authors demonstrated protection against APV challenge in turkey poults that were unable to seroconvert. These birds had been chemically bursectomised using cyclophosphamide (at a dose that had been shown to cause only B cell immunosuppression) yet they were equally resistant to clinical disease. However, it is likely that in laying turkey hens, the presence of neutralizing antibody in the blood is important for protection of the reproductive tract from viraemic viruses which may affect egg production [19].

Specific antibody responses to the fusion protein of APV have been demonstrated by Yu *et al.* [143]. These authors constructed a fowlpox recombinant virus that expressed the fusion protein of APV. After turkey poults were vaccinated twice with this recombinant, antibodies were detectable by ELISA and SN tests. In addition, partial protection from challenge with a virulent strain, two weeks after the second vaccination was observed. This work highlights the importance of the fusion protein for protective immune responses. The importance of a second surface glycoprotein for stimulating protective antibody production has been demonstrated by Cook *et al.* [91]. Although this

work was carried out *in vitro* it demonstrates the neutralising capacity of Mabs that recognize the G glycoprotein. Protection has also been demonstrated following intramuscular inoculation with recombinant N and M proteins however the mechanism of protection was not determined [144].

Work by Munir *et al.* [145] has provided key insights into many individual genes and pathways that constitute the host cells response to infection and highlight the complexities of host-virus interactions and resulting defense measures.

(ii) Maternal antibodies

Maternal antibody titres that have been vertically transmitted via the yolk sac will be directly related to the levels of circulating antibody in the parent bird. The presence of these antibodies does not interfere with live APV vaccination of turkey poults at a young age [141]. In addition maternal antibodies in turkey poults have failed to protect against virulent APV challenge [146]. These results also highlight the importance of the local and cell mediated responses in the role of protection.

(iii) Local immune responses

It appears that local immune responses in turkeys and chickens are important for protection against APV, since the virus replicates primarily in the upper respiratory tract. Following APV vaccination of day old chicks, local APV specific IgA antibodies in the lachrymal fluids have been demonstrated [127]. Khehra *et al.* [147] have also showed local, APV-specific IgA antibodies, in both lachrymal and tracheal washes of chickens and turkeys [147].

(iv) Cell mediated immunity

Jones *et al.* [142] demonstrated protection against an APV virulent challenge in turkey poults that were unable to seroconvert due to b-cell immunosuppression. This suggests that the cell mediated immune response provides the main resistance to infection of the respiratory tract with APV. Work of Ganapathy *et al.* [127] concurs with the suggestions of Jones *et al.* [142]. Following dual vaccination of SPF chickens with live APV and live NDV, birds were still protected from challenge with a virulent strain of APV irrespective of the levels of circulating APV antibodies (personal communication).

Murin & Kapur [145] have provided key insights into individual genes and pathways that constitute the host cells response to APV infection. They identified many genes in chicken embryo cells that were differentially regulated following in vitro infection with a subtype C virus and showed the transcriptional change in the interferon (IFN)-regulated class of antiviral genes to be the most striking. The induction of many IFN-stimulated genes (ISGs) increased as early as 2.5 hours pi and increased over a period of 96 hours. Increases in induction ranged from 2-61 fold. One of the most strongly induced proteins was the Mx protein. These proteins constitute a group of large antiviral GTPases which mediate their effect by sequestering viral nucleocapsids, therefore rendering them inaccessible for replication [148]. It is not known if the avian Mx protein has an antiviral effect towards APV or other avian respiratory viruses but activity against orthomyxoviruses, paramxyoviruses, rhabdoviruses, bunyaviruses and togaviruses have been shown using human MxA and mouse Mx1 proteins [149].

2.3.7 Transmission

When APV was first encountered its rapid spread among turkey flocks in England and Wales lead to initial assumptions that transmission must be airborne [102]. However, Cook *et al.* [113] reported that experimentally, virus did not transfer from infected poults to non-infected poults that were placed in the same room but in a different pen, even though air flow was favorable. Similar results have been reported by Alkhalaf *et al.* [150] using a subtype C APV. Conversely contact transmission has been demonstrated from affected to susceptible turkey poults, and by inoculation with filtered or unfiltered mucus, nasal washings or other material from the respiratory tract of infected birds [11, 151]. Cook *et al* [113] have demonstrated that APV was transmissible from infected to susceptible turkey poults that were in direct contact for a period of 9 days pi. There are reports of susceptibility to the virus in many other species including ducks [93], pheasants [94, 95], guinea fowl [95], ostrich [97] and herring gulls [98] and it has been suggested that the aquatic bird could be a possible carrier species. This is strengthened by similar studies on APV subtype C were it was found in domestic waterfowl [152] although in an Italian study of wild aquatic birds, APV was not detected [153]. The conflicting reports about APV infection in wild birds could suggest that they are susceptible to APV infection but may not play a role in transmission of infection.

No published evidence is available on vertical transmission even though high levels of virus can be detected in the reproductive tract of laying birds [122, 154].

2.4 Diagnosis

2.4.1 Clinical

Because the clinical signs of APV are not pathognomonic, demonstration of the virus or a rise in APV antibody titre is required for definitive diagnosis.

2.4.2 Viral antigen

(i) Isolation and demonstration of viral antigen

It is important to note that APV virus isolation is difficult from field samples. This is partly due its relatively poor replication characteristics in laboratory host systems, the frequency with which secondary organisms are isolated and the timing of isolation attempts [19, 155]. It is also laborious, time consuming and expensive. However, it may still be required for epidemiological studies, further characterization of a particular isolate or for use in collaboration with other diagnostic tests. Since replication is primarily confined to the upper respiratory tract, turbinates and trachea then samples from this area are preferred when attempting APV isolation. As mentioned, the timing of the isolation attempt is also important. Samples should be taken as early as possible after the initial stages of clinical disease. Experimentally both the chicken and the turkey have been shown to shed the most virus between 3 and 5 days pi [11, 12, 35, 113, 116, 122]. The systems of choice for primary isolation of APV from field material are either embryonated eggs inoculated via the yolk sac [35, 156] or chicken or turkey embryo TOC [11, 13, 35, 36]. The preferred method of choice is TOC, although no comparison between the two methods has been made. Cook and Cavanagh [157] suggest that the method of choice is likely to depend on the personal preference of the individual laboratories. However, for isolation of subtype C an important factor to consider is the inability of this virus to cause

ciliostasis [29] which means that the use of TOC is inappropriate for primary isolation. This may explain why attempts to isolate APV in the USA were initially unsuccessful.

After primary isolation the virus can be adapted to grow in a variety of cell cultures, including chick embryo fibroblasts (CEF) [137, 156], chick embryo liver (CELi) [140], and vero cells [35, 140]. However, it has been shown that multiple passages in tissue culture systems can quickly lead to attenuation of the virus [140]. Conversely up to 98 passages of virus in TOC did not result in any loss of virulence [140].

Recently two cell lines derived from grivet monkey kidney and foetal rhesus monkey kidney, have showed promise for growth of a subtype C vaccine strain [158], although the authors state that the CPE was not as clear as that observed in Vero cells and end points could only be determined after IF using hyperimmune anti-APV serum.

There are a number of techniques that can be used for demonstration of viral antigen in fixed and unfixed tissues and smears. However, these techniques have limited use for diagnostic purposes. The most widely used are IP, IF and less commonly immunogold staining [33, 36, 122-124, 133]. These techniques have generally been applied for replication and pathogenesis studies in both the turkey and the chicken. For the demonstration of viral particles EM has been used [10, 11, 13, 35] but usually to confirm the morphology of isolated virus. More recently molecular techniques have been developed to detect the virus. The most widely used of these techniques is the RT-PCR.

Although RT-PCRs are significantly more sensitive and rapid than conventional virus isolation methods [20, 157, 159] there are important things to consider. The durability of DNA, the powerful amplification procedure and high sensitivity of these tests means that there is a high risk of contamination. Moreover, the decision of whether to use a subtype specific or a generic-type PCR has to be made. Evidence that supports a subtype specific approach to PCR diagnosis of APV is that of a retrospective study [160]. This indicated that subtype A virus, previously only detected in South Africa and the UK had also been present in Germany in the late 1980s. However, to use a subtype specific approach would be to run the risk of overlooking new subtypes for which the chosen oligonucleotides would not detect. Therefore, the strategy suggested by Bayon-Auboyer *et al.* [161] to first use an N gene sequence-based RT-PCR for initial detection, followed in positive cases by a G gene sequence-based subtype specific RT-PCR for initial characterization would be more appropriate.

Cook and Cavanagh [157] suggest that the large L gene may also be a useful target for designing general PCR primers due to its conserved nature. A variety of RT-PCR techniques have been developed and have been extensively reviewed [157, 162-164].

2.5 Antibody detection

2.5.1 Virus neutralization

SN tests have been shown to have similar sensitivity to ELISAs [134] although their use is less frequent for general detection of APV antibodies. The SN test may be performed in a number of systems, including TOC [91] and cell cultures such as CEF, CEL, Vero and MA104 [10, 134, 138, 140, 165, 166] of which the

most commonly used is TOC. TOC which uses ciliostasis as an indicator of infection has the advantage that the virus requires no adaptation before it can be used in SN. However, this system is inappropriate for SN tests with subtype C as this virus does not cause ciliostasis. The indicator used in cell culture systems is the characteristic CPE that develops upon infection. Therefore, this system is useful for all known APV subtypes but the virus needs to be adapted to grow in the chosen cell line prior to SN.

2.5.2 Immunofluorescence

Baxter-Jones *et al.* [133, 134] used IIF, using APV infected TOCs, on serum samples from both experimentally and naturally infected turkeys. The results showed the test to be both sensitive and specific. Although these tests are useful for applications in research they are limited when testing large numbers of poultry sera for APV antibodies. For this reason these tests do not appear to have found favour in diagnostic laboratories. More recently an IF test with a 96 well, flat-bottomed microplate was developed to detect APV antigen in Vero cells [167]. Although in this test, the authors were looking for a method that would allow rapid detection of APV antigen, it may also provide a method for screening large numbers of serum samples, by IF, for APV antibodies.

2.5.3 ELISA

ELISAs have become, by far, the most commonly used serological method for detection of APV antibodies [23, 135, 137, 138, 152, 168, 169] and there are many commercial and in-house ELISAs that have been developed [10, 135-138, 170, 171]. Antigens for use in APV ELISAs have been prepared on different substrates such as TOC, CEF and Vero [10, 135-137]. Baxter-Jones et al. [34]

demonstrated similar antibody profiles when testing turkey sera using two different ELISA protocols and antigen that had been prepared in both TOC and CEF. It has been suggested that the choice of substrate for antigen growth appears to be a matter of personal preference [157]. However, higher virus titre can be achieved when using cell cultures; therefore, it may be preferential to use this method.

Although there are several substrates that can be used for antigen production, the choice of APV strain to be used in the ELISA is important. Eterradossi *et al.* [172] used two isolates of APV; a UK, subtype A isolate and a French, subtype B isolate as antigen in their ELISAs. Each antigen was tested against known positive sera from the UK (subtype A) and from France (subtype B). The results showed that each antigen was more efficient in detecting antibodies in sera that originated from the same country as the antigen; thus the authors suggest the importance of using viral antigens that have been prepared from strains originating in different geographical areas. Eterradossi *et al.* [23] also suggested that the choice of an inappropriate ELISA antigen could lead to a false diagnosis that indicates vaccine failure, or hinder early diagnosis of APV infection in both vaccinated and un-vaccinated turkeys. Moreover, it has been shown that vaccinal antibodies may not be detected if a heterologous APV strain is used as the coating ELISA antigen [23]. Therefore, it has been suggested that homologous antigens should be used in such circumstances.

A further important point is that ELISAs which incorporate either subtype A or B antigens have been found to detect subtype C antibodies very poorly [29]; again it was suggested that the homologous strain be used as coating antigen when testing sera by ELISA for antibodies to subtype C [29]. Such ELISAs have now

been developed [169, 170, 173]. Antigens for these ELISAs were prepared from both Colorado and Minnesota isolates.

A comparative study of commercially available ELISA kits also revealed considerable variation in sensitivity. Mekkes and De Wit [174] in testing three kits from different manufacturers, showed that while two detected an antibody response to live vaccination with both French and UK vaccines, one failed to do so. McFarlane-Toms & Jackson [175] found similar results. For reasons outlined above it is clearly desirable to develop ELISAs that are suitable for all purposes. One approach has been to develop novel ELISAs using recombinant proteins as antigen. Literature relating to this is discussed in section 2.6 below

2.6 Expression and use of recombinant viral proteins

An extensive variety of viral proteins, have been expressed in and from a range of prokaryotic and eukaryotic expression systems. These proteins have been used in a variety of studies including those directed at vaccine development (2.6.1), protein folding, transcription and replication (2.6.2), characterization of monoclonal antibodies and studies on immunity (2.6.3) and applications in ELISA development (2.6.4).

2.6.1 Recombinant proteins in vaccine development

Delivery of APV subtype A fusion protein to turkeys has been achieved using fowlpox recombinant virus Qingzhong et al [143] resulting in partial protective immune response and induction of antibodies detectable by ELISA and virus neutralization.

For APV subtype C, antibody responses to the fusion protein have also been demonstrated in turkeys Tarpey et al [176] following two intramuscular injections with DNA plasmids carrying the encoding gene. Although the authors reported no clinical protection following homologous challenge, they demonstrate cross reaction of antiserum to both A and B subtype APV's. Therefore, although not discussed by the authors, they might have inadvertently identified the fusion protein as a useful antigen candidate for single recombinant ELISAs that detect antibodies against A, B and C subtypes. Similar DNA vaccine studies have been undertaken using both the fusion and the nucleocapsid genes of subtype C [177]. In contrast to the findings of Tarpy et al [176] the authors reported significant homologous protection against challenge in turkeys that received DNA plasmids containing the fusion gene. These discrepancies might be explained by differences in the virulence of challenge virus. However, Kapczynski et al [177] made no reference to, or discussion of the work of Tarpy et al [176] which had been undertaken two years previous.

2.6.2 Protein folding, transcription and replication

Nucleocapsid proteins of paramyxoviruses have been the focus of many studies due to their importance during replication [178-181]. Interestingly Kho et al [182] demonstrated the ability of *E.coli*-expressed Newcastle disease virus N protein to self-assemble into the herringbone-like structure which morphologically resembled the authentic structures described by Alexander et al [183]. Later Kho et al [181] produced several deletion variants in *E.coli,* which helped to identify amino acids important for proper formation. Prior to this study much structural and functional information on this particular protein had been derived from other members of the paramyxovirus family in similar studies. These authors also

demonstrated that deletions further from the c' terminal end tended to form longer particles.

Fearns et al [184] also used the nucleocapsid protein in conjunction with the phosphoprotein of respiratory syncytial virus to investigate a current model for RNA synthesis of nonsegmented negative-strand viruses. These authors expressed nucleocapsid and phosphoproteins from transfected plasmids carrying the genes and evaluated their effects on the replication of RSV minireplicons. Essentially the authors demonstrated that the nucleocapsid protein was required for RNA replication and that replication was stimulated by increased levels of nucleocapsid protein.

2.6.3 Characterization of monoclonal antibodies and studies on immunity

N protein of subtype C APV expressed from a transfected plasmid in HEp-2 cells [185] helped to characterize six monoclonal antibodies prepared against this virus. Yu et al [185] showed that although none of these antibodies neutralized APV infectivity at a detectable level, they all recognized both denatured and nondenatured forms of the N protein, thus suggesting that these antibodies recognized structurally independent epitopes. A similar study characterizing monoclonal antibodies against avian reovirus (ARV) with a baculovirus-expressed recombinant ARV sigma C protein was recently undertaken by Hsu et al [186]. These authors also demonstrated conformation-independent binding of the monoclonal antibodies to the recombinant protein.

Analysis of immune responses to baculovirus and vaccinia virus-expressed RSV surface proteins F and G has been undertaken [187, 188]. Werle et al [188] using baculovirus-expressed full length F protein or a fragment of RSV F protein were able to identify at least two neutralizing epitopes within this region. Olmsted et al [187] reported that immunization of cotton rats with vaccinia-expressed F protein stimulated almost complete resistance to replication of RSV upon challenge and that resistance offered by F exceeded that induced by vaccinia-expressed G protein.

2.6.4 Applications in ELISA development

Recently, individual, recombinant viral proteins have been tested for their suitability as ELISA antigen candidates for APV subtype C [2, 3, 63]. Both sandwich and indirect ELISAs using subtype C nucleocapsid and matrix proteins respectively, provided more specific and sensitive tests compared with routine ELISAs [2, 3]. Lou *et al.* used the SH protein of the same subtype and developed an ELISA that was suitable for detecting subgroup-specific antibodies in turkeys. They suggested that this test could be used for serologically-based differential diagnosis of APV and hMPV infections. The authors state that the SH protein was chosen as a likely candidate for a subgroup-specific serodiagnostic reagent because it demonstrated extensive amino acid variation between subtypes A, B and C and hMPV. Other APV proteins or regions of proteins share the same properties or conversely, maintain conserved sequences between subtypes. This means that theoretically, different recombinant proteins have the potential to detect subtype specific antibodies or antibodies common to all subtypes, when used as antigen in ELISAs. Such ELISAs have also been developed and tested for other members of the paramyxoviridae family [1, 4-7] each demonstrating

increased or equal sensitivity and specificity with conventional ELISAs. Chen et al [8] highlighted some important advantages of using recombinant proteins over whole virus-infected cell extracts as antigen. These include safety (being non infectious) and having the potential to be modified rapidly in respond to new viral strains. In this study, the authors compared sensitivities of infectious bronchitis virus ELISAs using baculovirus and *E.coli*-expressed nucleocapsid protein as antigen. Surprisingly, they found that N protein purified from *E.coli* was more sensitive to anti-IBV serum than that purified from insect cells even though *E.coli* expressed N protein lacked phosphorylation. This may suggest that IBV antibodies detected in the ELISAs recognized structurally independent epitopes, such as described by both Yu et al and Hsu et al [185, 186] following their studies on APV and ARV respectively. However, it could also suggest that epitopes reacting in the ELISAs of Chen et al [8] are not dependent on phosphorylation for their formation.

2.7 Control

2.7.1 Management and treatment

Management factors play an important role in the severity of clinical signs and therefore the severity of APV infection in commercial poultry, especially turkeys. Conditions such as high stocking densities, poor litter quality, inadequate ventilation and temperature control, multi-age stock and secondary pathogens all exacerbate infection [21, 131, 154, 189]. In general, any procedure that causes stress to the birds can influence the severity of infection. Gough and Jones [190] suggest that disinfection of delivery and catching crews, equipment and feed trucks should also be routine practice to reduce the introduction and spread of APV onto poultry farms.

Problems of secondary infections can be reduced through the use of antibiotics. Initially medications aimed at alleviating secondary infections had only limited success [21] but later, Hafez *et al.* reported success in using enrofloxacin [191]. Most recently Munir *et al* [192] have provided interesting results that could lead to the development of novel RNA interference (RNAi) based antiviral prophylactic treatments. These authors showed that double stranded short interfering RNA (siRNA) molecules that corresponded to a subtype C, APV P gene broadly reduced the expression of P and other viral proteins and also led to the inhibition of subtype C virus replication *in vitro*. Moreover, this technique is being increasingly utilized to inhibit the replication of other viral pathogens, including HIV-1 [193], influenza [194, 195], hepatitis B & C [196, 197], poliomyelitis [198], foot and mouth disease [199] and severe acute respiratory syndrome [200]. It is important to note that efficient means for *in vivo* APV P siRNA delivery have not yet been developed.

2.7.2 Vaccines

The main approach for control of APV infection is through the use of both live-attenuated and killed vaccines [35, 140, 141, 201-204], both of which are available commercially. Live attenuated vaccines have been successfully produced in a variety of cell cultures and have been reported to confer protection against experimental infection [35, 140, 141, 201]. In the field, the results have been generally good following spray, aerosol or drinking water administration although problems have arisen possibly due to a lack of control over simultaneous exposure of the entire flock to the vaccine via these methods. Moreover, because of the genetic instability of RNA viruses, live vaccines that have been attenuated through minimal coding sequence changes after multiple

cell culture passages have a tendency to revert to virulence. Therefore if birds only become exposed to the vaccine following several back-passages, then exposure may be to a virulent revertant or possibly to a virulent sub population in the vaccine. Naylor and Jones [17] demonstrated, a virulent sub population in a prototype live-attenuated vaccine using *in vitro* and *in vivo* screening techniques and suggested that this could account for the occasional disease seen in young poults following multiple back passage of the vaccine. At the time of these studies it was difficult to determine if these virulent viruses were indeed vaccine derived. However, in a recent study Catelli *et al.* [205] have demonstrated the reversion to virulence and extended persistence of the same live-attenuated vaccine and confirmed its vaccine origin.

Studies have shown that good cross protection occurs following vaccination with subtype A and B vaccines against challenge with either subtype [23, 172, 201, 206]. Vaccines prepared from either A or B subtypes have been shown confer protection against the Colorado subtype C virus [20].

Vaccination with live attenuated vaccines alone will not provide complete protection in adult birds; therefore, an oil-adjuvanted inactivated vaccine is administered after the initial live vaccination. Gough and Jones [190] suggest a typical vaccination programme in turkeys as: live subtype A or B or both at day-old using a coarse spray, repeated at 7-10 days and again at 4-6 weeks. Breeding stock would also receive inactivated vaccine at 16-20 weeks.

Studies in turkeys on the immunity to an APV vaccine following in ovo vaccination have also been reported [207] and the results indicated that this route of vaccination has several advantages over conventional methods including, more accurate administration of the vaccine that can be done in the hatchery, possible

multi vaccination in a single dose and less handling of the birds resulting in reduced stress. A recent preliminary study has indicated that inoculation of poults with a single dose of a cold-adapted subtype C strain of APV at 1 week of age has provided protection until 15 weeks of age [208]. However, the authors stress that trials using larger sample numbers would be needed to determine the application of these results in the field.

As mentioned earlier the genetic instability of RNA viruses and the minimal coding sequence changes that result from passage [205] means that they can readily revert. The recent developments of recombinant subunit and deletion mutant viruses [72] mean that reversion is much less likely. Whether these recombinant viruses provide protection and become useful vaccines is yet to be established. Other recombinant vaccines that incorporate specific immunogens, such as the F protein, in fowl pox virus [143] have had some success. This vaccine was shown to induce APV antibodies and provided some protection in experimental turkeys when challenged [143]. Another interesting approach was the development of an APV virosome vaccine [209] which also conferred protection in experimental conditions. These types of vaccines are designed to contain viral membrane proteins within a liposome complex but do not possess genetic nucleic acid. Therefore they are as safe as inactivated vaccines and are still able to attach and fuse with host cells [209].

Chapter 3

General materials and methods

Contents **Page**

Chapter 3

General materials and methods

This section describes routine procedures undertaken during this work. Details of specialized procedures are given in the relevant chapters.

3.1 Glassware

All items of glassware were cleaned as follows before use.

3.1.1 Treatment of contaminated glassware

Glassware was machine washed using detergent (Neodisher GK, Chemische Fabrik Dr. Weigert, Hamburg, Germany) then rinsed twice with tap water then three times in ultra pure water. Sterilisation was by autoclaving at 121^{0}C for 30 minutes. Flasks and measuring cylinders were closed with aluminium foil and sterilized in a hot air oven at 160^{0}C for 2 hours.

After use all glassware contaminated with infectious material was soaked overnight in Virkon (ANTEC International Sudbury, Suffolk, England), washed and autoclaved as above.

3.2 Overview of cloning, expression and purification using a prokaryotic system

Genes were amplified using PCR techniques, ligated into various plasmid vectors and inserted into *E.coli* by heat shock transformation. After bacterial culture DNA was extracted. The exact nucleotide sequence of constructs was determined before sub-cloning into specialized *E.coli* expression cells.

3.2.1 Expression

Foreign genes were placed under the control of an inducible phage-based T7 promoter which prevented expression of potentially toxic proteins, which might affect E Coli replication, prior to induction by IPTG. Binding of IPTG to a repressor induced conformation changes which lead to its dissociation followed by gene transcription and expression. (Figure 5 steps 1 to 4).

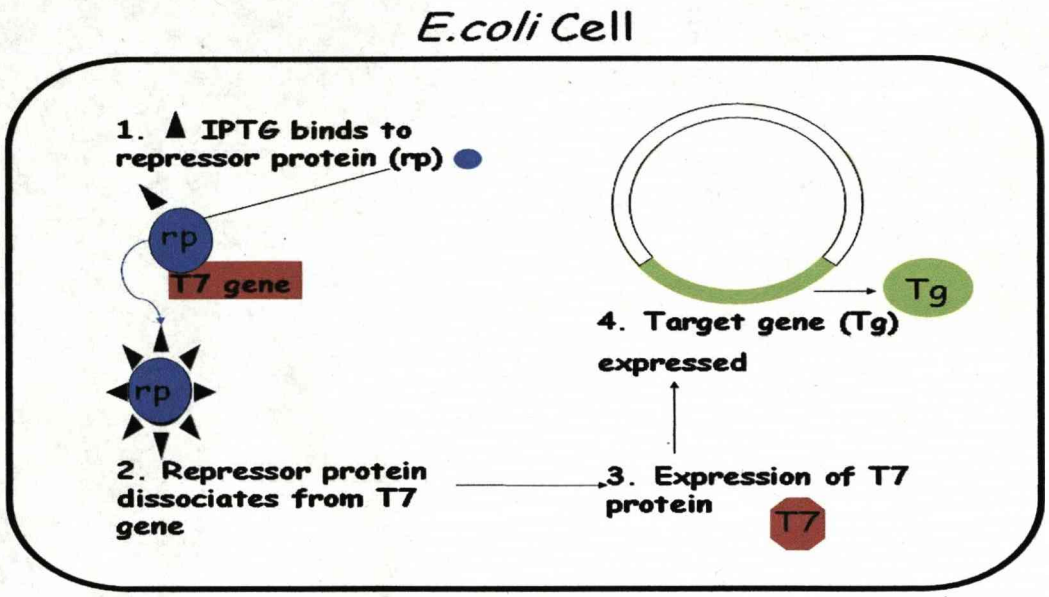


Figure 5. Steps in target gene expression after induction with IPTG

3.2.2 Purification

DNA sequences coding for a polyhistidine tag were added to genes to enable proteins to be purified using matrices containing immobilized Ni 2+ ions.

3.3 Construct development and analysis

3.3.1 Polymerase Chain Reaction (PCR)

Pfu polymerase (Stratagene, Amsterdam, Netherlands Cat no. 600250) was used to amplify genes for cloning and expression and was added at 80^{0}C to the

reaction mixture to avoid mispriming and subsequent extension from unintended regions of sequence. For general diagnostic PCRs taq polymerase (Promega, Southampton, UK Cat no. M1861) was used. Both followed current recommended protocols.

Pfu PCR reaction mixture 50ul in 500µl PCR-tube

5µl Pfu 10x buffer

0.5µl dNTP solution (10mM per nucleotide)

5 pmols each oligonucleotide primer

40µl double processed tissue culture water (Sigma W3500)

0.1 µg template

50µl mineral oil (Sigma M-8410)

1µl Pfu Enzyme

Taq PCR reaction mixture 25ul in 500µl PCR-tube

2.5µl Taq 10x buffer

1.75µl magnesium chloride solution

0.5µl dNTP solution (10mM per nucleotide)

0.25µl Taq DNA polymerase (5U/µl)

17µl double processed tissue culture water (Sigma W3500)

5 pmols each oligonucleotide primer

0.1 µg template

50µl mineral oil (Sigma M-8410)

3.3.2 Site-directed mutagenesis (SDM)

This was used to correct any nucleotide sequence errors or to introduce specific sequence changes to developing constructs, details of which are dealt with in the relevant chapters.

(i) Theory

DNA replicated in most E Coli strains used for cloning is methylated while DNA copied from it using available polymerases is not. Restriction endonuclease Dpn 1, (Invitrogen cat no. 15242-019) digests only methylated DNA, so after digestion, the desired copied DNA construct can be used without contamination with the original template (See Figure 6).

(ii) SDM reaction

5µl 10x PfuTurbo buffer (see 3.2 above for product information)

20pmols each oligonucleotide primer

1µl dNTP solution (10mM for each nucleotide)

0.1 µg template

38µl double processed tissue culture water (Sigma W3500)

1µl PfuTurbo (2.5U/µl) added at 80^0C

(iii) SDM PCR cycle

1. 80^0C hot start
2. 95^0C 30 seconds
3. 95^0C 30 seconds
4. 55^0C 1 minute (temperature varied depending on the T_m Values [210])
5. 68^0C at 2 minutes / kilobase (Kb) of template

Steps 3-5 were repeated between 12 and 18 times

Hold temperature was 8 ^{0}C

(iv) *Dpn* 1 digestion

0.5µl of Dpn 1 (2-8 U/µl) in 20µl SDM product, incubated at 37^{0}C for 4 hours minimum.

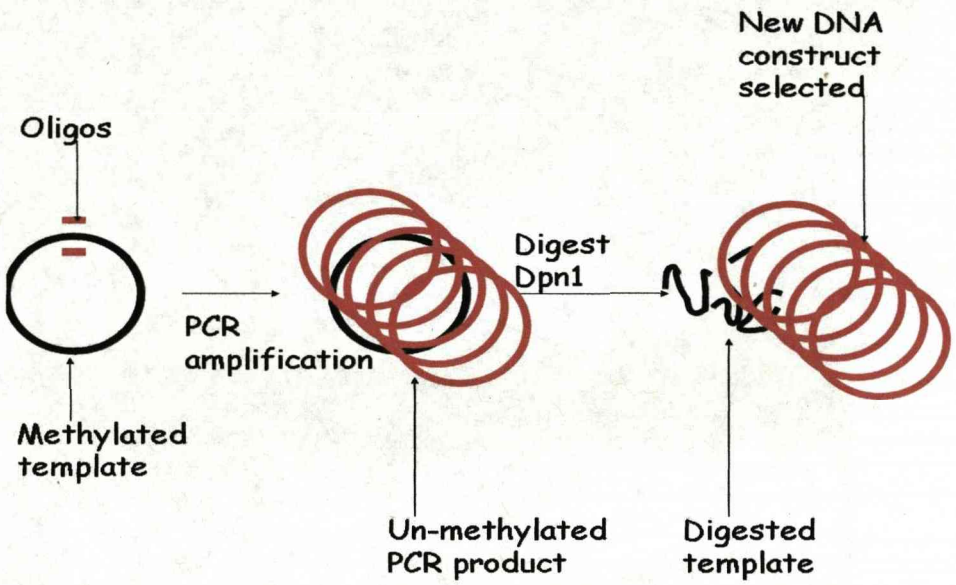


Figure 6. Site-directed mutagenesis using the methylated DNA specific restriction endonuclease, Dpn 1.

3.3.3 Ligations

Circularizing of blunt ended PCR products and ligation of inserts into vectors were carried out using Fermentas ligase (T4 DNA ligase # EL0013) using the recommended ligation protocol except that the reaction was performed at 14^{0}C for twelve hours.

3.3.4 *E.coli* Transformations

Transformations of library efficiency DH5α competent cells (Invitrogen # 18263-012, stock cells for maintaining plasmids) and BL21 (DE3) (Invitrogen # C6060-10 expression cells) were done following supplier's protocols.

A volume of 50-100µl of transformed cells was plated on LB agar (Sigma, Poole, UK #L.2897) plates containing antibiotic appropriate to the vector. Either ampicillin was used (Sigma # A.0166) at a concentration of 100µg/ml or kanamycin (Invitrogen, UK) at a concentration of 15µg/ml. Plates were incubated at 29-30^{0}C.

3.3.5 Plasmid preparations

Where a high level DNA purity was not needed a crude method was used. In all other situations an appropriate Qiagen kit was used. .

Minipreps using commercial Qiagen miniprep kit Cat. No. 27104

Preparations followed the recommended protocol using a 5 ml starter culture. DNA was eluted with water (Sigma W3500)

Crude Standard technique

Individual *E.coli* colonies were picked from agar plates into 3ml of LB broth (Invitrogen # 1085521). Cultures were incubated overnight at 30^{0}C with agitation. 1.5ml of culture was placed into a 1.5ml centrifuge tube and spun at 13.2 x 1000rpm for 30 seconds. The pellet was re-suspended in 300µl of TENS buffer (see Appendix) and vortexed until the suspension was viscous. A volume of 150µl of N_aAC pH5.2 3M was added, vortexed and centrifuged at 13.2 x 1000rpm

for 2minutes. The resultant supernatant (400µl volume) was mixed with 900µl of 100% ethanol and incubated at –20^{0}C for approximately 15 minutes. The samples were centrifuged at 13.2 x 1000rpm for 6 minutes and the supernatant discarded. Pellets were washed in 70% ethanol, air dried and re-suspended in 50µl of double-processed tissue culture water (Sigma W3500) containing RNase (Sigma # R-4642) at a concentration of 60µg/ml. Preparations were kept at –20^{0}C and thawed for use as required.

3.3.6 Restriction Endonuclease (RE) digestions

Digestions were used to prepare plasmids and inserts for ligations and after cloning to map new constructs to check their identity. In general digestions followed supplier's protocols.

3.3.7 Sequencing

Plasmid and PCR DNA was sequenced by Advanced Biotechnology Centre (ABC), Imperial College, London. PCR DNA templates were treated with exonulease 1 and shrimp alkaline phosphatase (Exo / Sap Appendix) respectively to remove PCR primers and excess dNTPs, which would otherwise interfere with sequencing reactions. Nucleotide sequence data were analysed using Chromas version 1.45, Generunner version 3.05 and Bioedit version 5.0.9.

3.3.8 Agarose gel electrophoresis

Agarose gels of concentrations between 0.8 and 2% w/v were prepared in TBE buffer.

Three parts of sample were mixed with one part of DNA load buffer (see Appendix) in a suitable volume prior to loading. Following electrophoresis, gels were stained with ethidium bromide solution (see Appendix). A molecular weight (MW) standard was used at all times (Appendix)

3.4 *E.coli* protein expression

3.4.1 Expression

Three derivatives of the *E.coli* strain DE3 were used for expression: 1. BL21 (DE3), 2. BL21 (DE3) pLysS and 3. BL21 (DE3) pLysE were obtained from Invitrogen, UK.

Colonies that contained the desired plasmids cultured in LB broth (Invitrogen # 1085521) overnight at 30^0C. These cultures were used to seed fresh cultures in LB broth, at a dilution of 1:50 and then incubated again at 30^0C until after approximately 3 hours reaching mid-log phase at a reading of 0.4 OD_{600}. Cultures were induced with IPTG at concentrations between 0.5mM-1mM and incubated for an additional 1-4 hours. Induced cells were analysed for the expression of desired proteins.

3.4.2 *E.coli* cell lysis

50ml of Cell culture was centrifuged at 1,500 x g for 10minutes

Pellets were re-suspended in the following lysis cocktail followed by 15 minutes sonication.

Lysis cocktail

20µl protease inhibitor (Sigma, P 8849)

150µl triton x (Sigma, T8787) (10% in PBS)

30µl (100mg/ml in distilled water) lysozyme (Sigma, L6876)

7.5µl DNAse (Sigma, D5025)

Made up to 1ml in the following buffer:

Lysis buffer

0.535g $NaH_2PO_4 + 2H_2O$

3.07g Na_2HPO_4

8.77g NaCl

0.7g Imidazole (Sigma 10250)

Made up to 500ml with distilled water.

3.4.3 Protein purification

Proteins were purified using Ni-NTA Spin columns (Qiagen Crawley, UK catalogue number 31314) using supplier's protocols. However, all cell lysates were filtered using 0.2µm syringe filters (SLS scientific laboratory supplies, UK) before application.

3.5 Baculovirus expression

3.5.1 Overview

When linear baculovirus DNA lacking certain essential sequence is transfected into insect cells together with a transfer plasmid containing those sequences, homologous recombination occurs and virus is produced In this study, Sf9

insect cells (Invitrogen, UK) were transfected with plasmid vector pBlueBacHis2 (Invitrogen , UK) to which genes of interest (GOI) had been added.

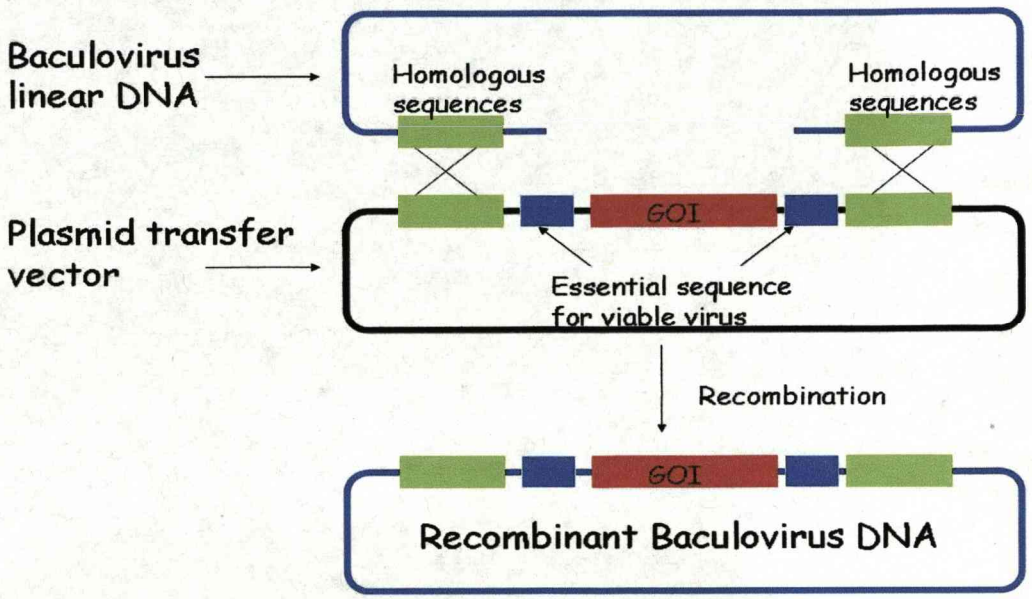


Figure 7. The generation of recombinant baculovirus DNA.

3.5.2 Insect cell cultures

Sf9 cells were maintained in various sized cell culture flasks (NUNC, Denmark) following Invitrogen's recommended protocols (Insect cell lines manual, version K 25-0127).

3.5.3 Baculovirus plasmid vectors

pBlueBacHis2 (Invitrogen, UK) plasmids were used. Methods described under section **3.3** were used to develop these vectors.

3.5.4 Transfections and plaque purifications

Invitrogens Bac-N-Blue transfection and expression instruction manual (version M, 25-0109) was followed throughout.

3.6 Protein analysis

3.6.1 Polyacrylamide gel electrophoresis (PAGE)

Expressed proteins were analyzed using sodium dodecyl sulphate PAGE (SDS-PAGE) as described by Laemmli [211]. Gels were stained with coomassie brilliant blue (see appendix).

Electrophoresis apparatus was the SCIE-PLAS (Warwickshire, UK) V10-WCDC with 1mm combs.

(i) Gels

Gels are formed by polymerizing an acrylamide monomer into long chains and cross linking these chains using N, N'-methylene bisacrylamide (bis). The percentage of SDS-PAGE gels depends on the ratio of acrylamide monomer to bis. Applications are as follows:

3% gels- isoelectric focusing, 5-30% - molecular sieving and

gels at 30% can be used for molecules with a relative molecular mass (M_r) as small as 2000. Gel preparation and recipes are described in the appendix.

(ii) Protein sample preparation and loading

See Appendix

(iii) Molecular weight markers

See Appendix

3.6.2 Western blots

These were used for serological identification of captured proteins.

Following SDS-PAGE proteins were transferred electrophoretically onto a nitrocellulose (NC) membrane by virtue of the negatively charged SDS that was bound to the proteins. Captured proteins were then probed with antibodies. Bound antibodies can be detected using an enzyme-conjugate which catalyses a reaction with diamino benzidine (DAB, Sigma, D5905) substrate to give an insoluble stain. Molecular weights (MW) were determined by comparing relative mobilities with known standard markers.

(i) Electrophoretic protein transfer

After running a standard SDS-PAGE gel the positions of the molecular weight markers and the bromophenol front was measured. This allowed molecular weights of unknown samples to be calculated post western blot.

One of the glass plates covering the gel was removed and NC paper and filter papers were cut to the exact size of the relevant section of the gel.

Filter papers were stacked into three lots of six and soaked from one end in relevant buffer, these were either the anode 1, anode 2 or the cathode buffer (Electrode transfer buffers Appendix).

The western blot sandwich was constructed as follows:

							Gloved hand				
							Anode 1				Cathode
					Anode2		Anode 2				gel
		NC			NC		NC				NC
Gel	->	Gel	->		Gel	->	Gel	->invert			Anode 2
Glass		Glass			Glass		Glass				Anode 1

Naylor, [212]

The above assembly was then placed onto the lower cathode plate followed by the upper anode plate as shown in Figure 8

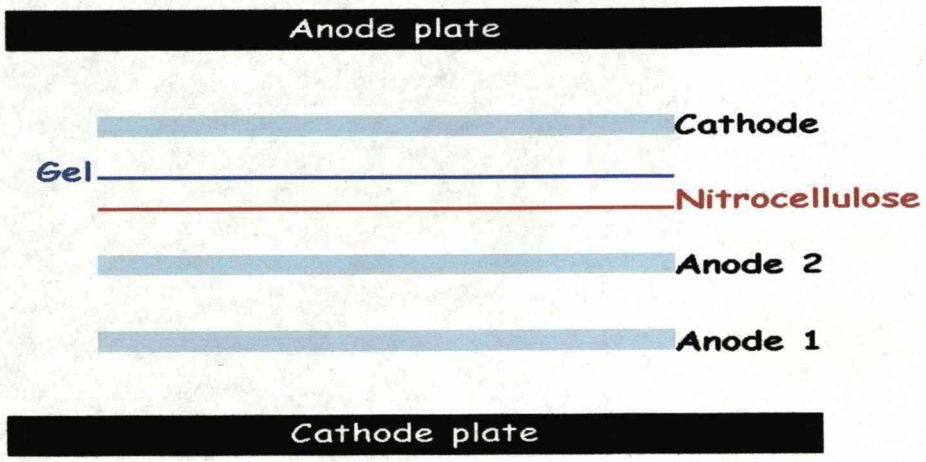


Figure 8 Western blot sandwich.

Gels were blotted for 2 hours at 80 milliamps (mA)

(ii) Development of nitrocellulose membranes

Small sealable 16mm x 10mm polythene bags were used for the development of NC membranes immersed in relevant solutions. Bags were agitated every 10-15 minutes over a 1 hour incubation period to prevent local starvation of constituents. Between solutions the membranes were washed a minimum of 3 times in phosphate buffered saline (PBS, see Appendix) containing 0.05% $Tween_{80}$ detergent (Sigma, Poole, UK).

The sequence of solutions used was as follows:

APV western blot

1. Overnight blocking step at 8^0C of 1% bovine serum albumin (BSA Sigma, Poole, UK) in PBS containing 0.05% $Tween_{80}$ (PBS/Tween).

2. Polyclonal serum diluted 1/100 in PBS/Tween for 1 hour

3. Goat anti turkey (GAT) horse radish peroxidase conjugate (ISL, immune systems limited, Paignton, UK) diluted 1/5000 in PBS/Tween for 1 hour

4. Substrate buffer (see Appendix) for 1 minute

5. DAB solution (see Appendix) until bands appear, usually between 5-15 minutes.

6. Reaction stopped by immersing in distilled water for 30 minutes.

His tag western blot

1. Same as 1. above

2. Monoclonal, anti His, horse radish peroxidase (HRP) conjugated antibody (Alpha Diagnostics Cat no. HISP12-HRP) diluted 1/10,000 in PBS/Tween for 1 hour.

3. Follow steps 4-6 above.

3.6.3 ELISAs

Some aspects of these procedures were consistent throughout the thesis, however many parameters were changed depending on the application. Therefore, ELISAs are explained in relevant chapters. Details of the antisera used in these tests are given below.

(i) Subtype A antiserum: At 75 days, SPF chickens were inoculated with a virulent subtype A APV and then again at 96 days by ocular route. Birds were

bled seven days post second infection. Each bird received 0.1ml of 5.5 $\log_{10}$ CD_{50} (Kindly provided by Dr Kanan Ganapathy, Liverpool University)

(ii) Subtype B antiserum: At 49 days, SPF chickens were inoculated with a virulent subtype B APV by ocular route and birds were bled ten days later. Each bird received 0.1 ml of 4.0 $\log_{10}$ CD_{50} (Kindly provided by Dr Kanan Ganapathy, Liverpool University)

(iii) Subtype C antiserum: Day old chicks were inoculated with a Colorado strain subtype C APV by ocular route and then again at 3 weeks by intravenous route. Birds were bled 24 days later (Kindly provided by Dr Fengsheng, Intervet UK)

(iv) IBV antiserum: This was generated using the same method described for subtype C APV antiserum (Kindly provided by Mrs Karen Worthington, Liverpool University)

Chapter 4

Development of a prokaryotic expression vector: Design, cloning and expression of APV genes N, P and F and the GFP gene

Contents **Page**

Chapter 4

Development of a prokaryotic expression vector: Design, cloning and expression of APV genes N, P and F and the GFP gene

4.1 Introduction

At Liverpool several plasmid vectors have been designed which readily accept APV genes and were designed for the first rescue of an APV from recombinant DNA [72]. The plasmid developed from pUC18 in that work, became the chosen vector for the prokaryotic expression work described herein. Although this plasmid was an efficient acceptor of APV genes it required some extra elements (see Figure 9) to enable expression of his tagged proteins in *E.coli*. The new plasmid was named p18smahis.

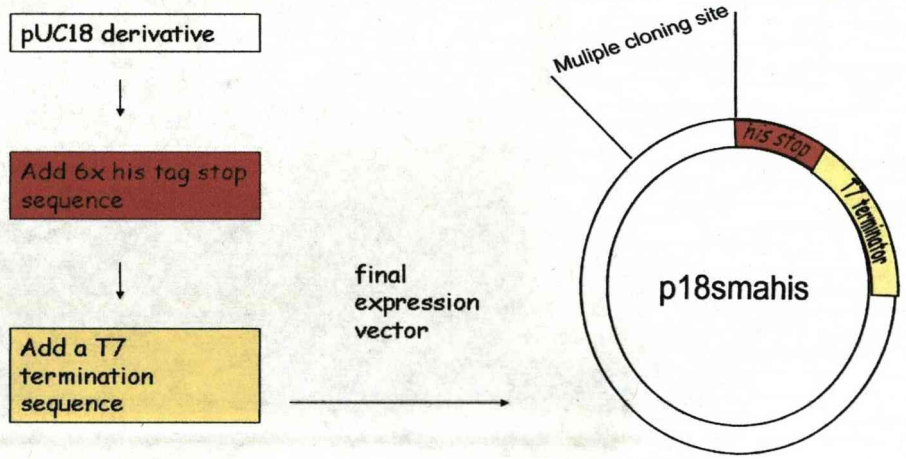


Figure 9. Manipulations of a plasmid derived from pUC18, to generate a new expression vector: p18smahis.

All genes were amplified from existing clones using new PCR primers which introduced a T7 promoter followed by a ribosomal binding site (RBS) sequence to the start of the gene, and removed the stop codon from the trailer (Figure 10).

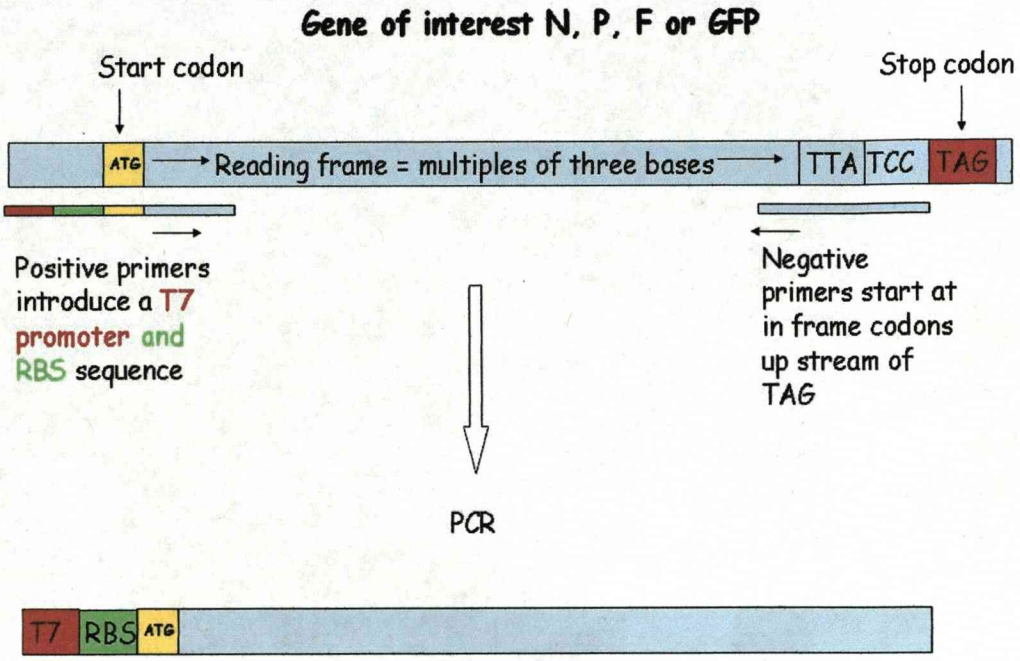


Figure 10. Amplification of GFP and APV genes: Removing the stop codons and adding T7 and RBS sequences.

New stop codons and his tag regions were introduced to these PCR products by ligating (Section 3.3.3) into p18smahis (Figure 11).

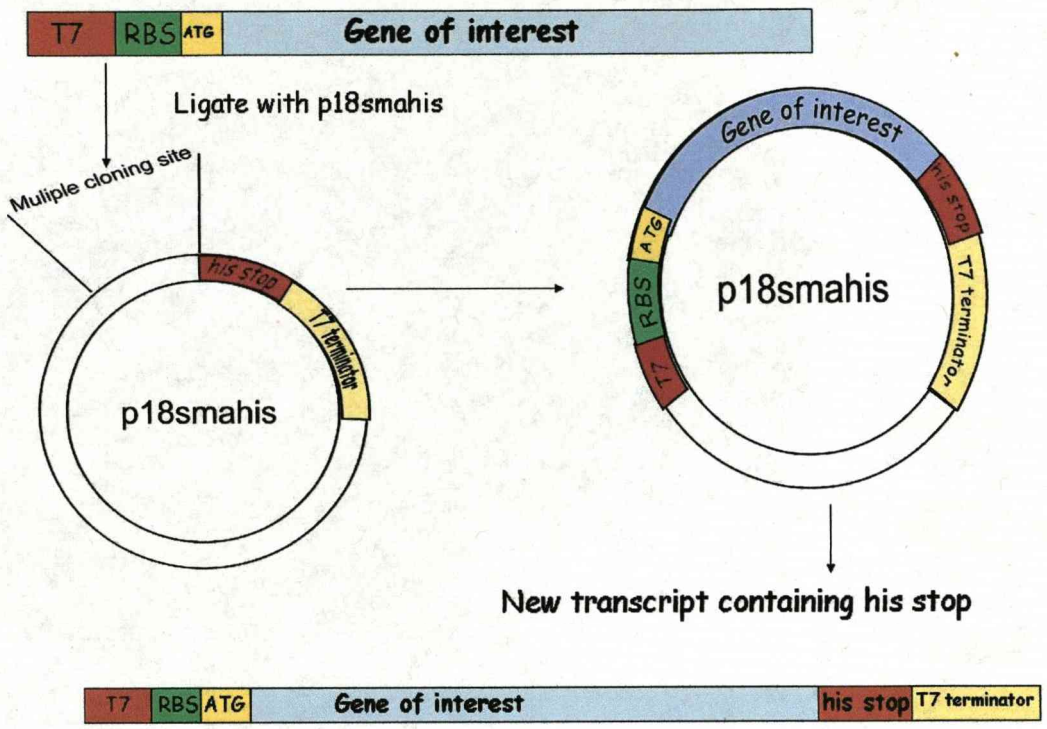


Figure 11. Generation of GFP or APV transcripts that contain a his tag region.

Clones that contained the exact sequence and reading frame of a particular inserted gene were sub-cloned into specialised *E.coli* cells for expression. These genes had been placed under the control of a T7 promoter and therefore required T7 polymerase for transcription. *E.coli* strain BL21 (DE3) express T7 polymerase following induction with IPTG and can therefore transcribe such genes. However, because some proteins may be toxic to *E.coli* control measures were needed which included lowering the concentration of IPTG and reducing the time scale over which expression is induced.

GFP was sub cloned first because expression could be rapidly detected. Slides were prepared using induced and non induced wet culture and fluorescence / expression was compared using a UV microscope.

F, N and P constructs used the same system and expression was determined using SDS-PAGE. These recombinant proteins all contained a his tag region for capture and purification using Ni-NTA spin columns. Again SDS-PAGE was used for analysis.

4.2 Materials and methods part 1: Development of a prokaryotic expression vector

Primers P18es2.6- **5'**-GGGGGAATTCTGCGCGGAAC-**3'** and p18es800+ **5'** GGGGAATTCGCAGGAAAGAACATG **3'** were used to amplify a pUC18 derivative in a Pfu PCR. This generated a blunt ended linear product which, when circularized using T4 DNA ligase (see 3.3.3), produced a vector containing desired restriction enzyme sites. The ligated DNA was treated with *Dpn* 1 restriction enzyme to digest the original template then used to transform library efficiency DH5α competent cells (see 3.3.4). Subsequent bacterial colonies were screened using the standard Taq PCR protocols (see 3.3.1). Products from this procedure were analysed using agarose gel electrophoresis (see 3.3.8). Plasmids were extracted from positive colonies using a Qiagen miniprep kit as described in section 3.3.5 then prepared for sequencing. Those with the correct sequence were digested with a Sma1 restriction enzyme (see 3.3.6) and ligated with a double stranded DNA segment containing a his tag region followed by a translational stop codon. This segment was generated by annealing approximately 1μg of primer sma-his-pos **5'**-GGGCATCACCATCACCATCACTA G-**3'** and 1μg of primer sma-his-neg **5'**-CTAGTGATGGTGATGGTGATGCCC-**3'** in a PCR block using the following program: 94^{0}C for 3 minutes then 40^{0}C >30minutes. The transformation, screening, plasmid preparation and sequencing procedures described above were repeated.

Adding the T7 termination sequence

Mfe1 and EcoR1 restriction endonucleases have different recognition sequences but leave the same 5' AATT overhang. After digestion the 2 sites can be ligated and because both recognition sequences are lost, it will not be recognized by

either enzyme. These properties were pivotal in the T7 termination cloning strategy illustrated in Figure 12.

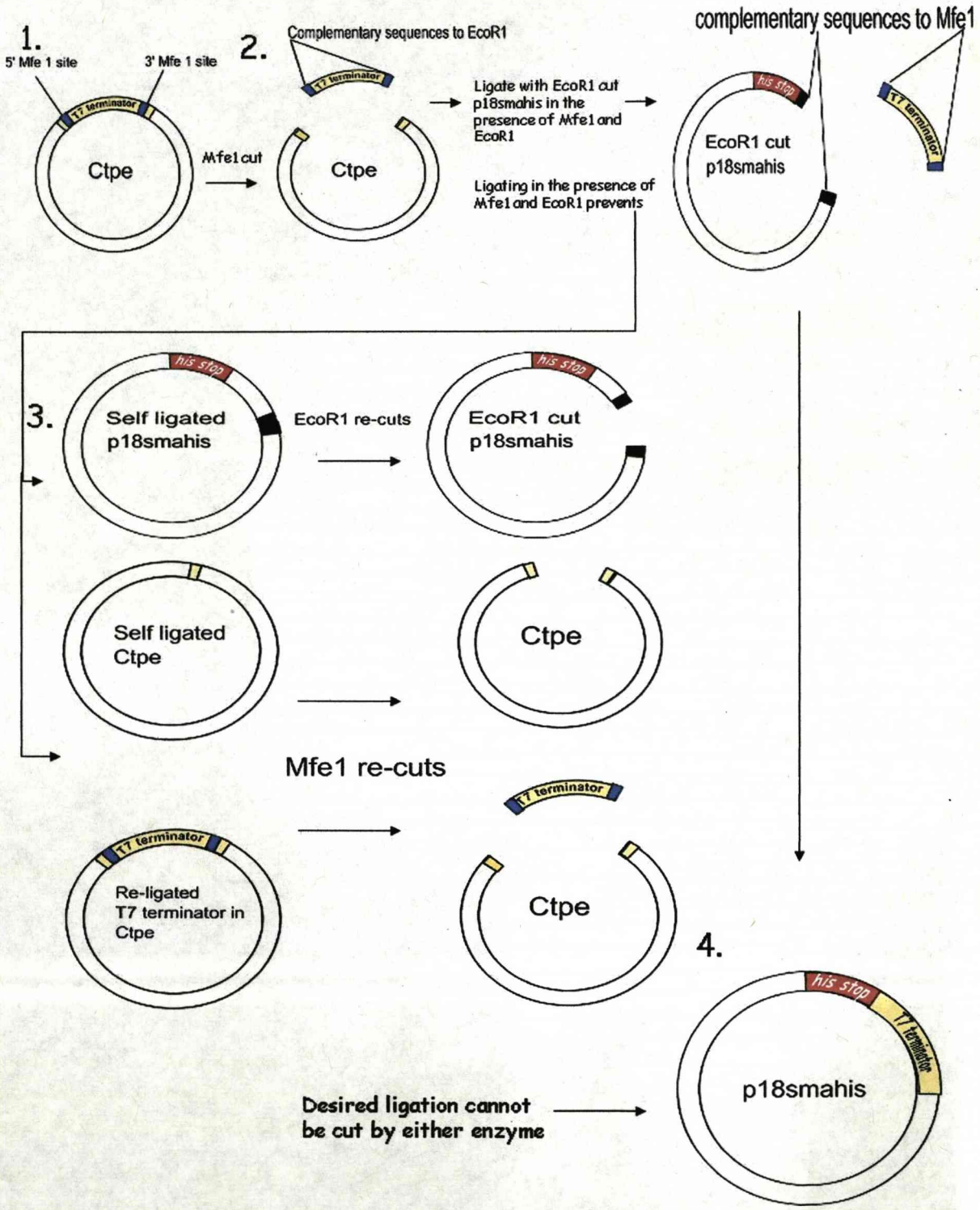


Figure 12. Adding a T7 termination sequence to p18smahis.

A kanamycin resistant plasmid (Ctpe) that had been developed by Naylor et al. [72] was ligated with a blunt ended, T7 termination PCR product. This contained Mfe1 sites at both the 5' and 3' ends (Figure 12. step 1.). Transformed DH5α colonies were cultured in LB broth containing 100µg/ml of kanamycin and plasmids were extracted using a Qiagen plasmid miniprep kit. The T7 termination sequence was excised from the purified plasmid by digesting with Mfe1 leaving a product with complementary end sequences to that of EcoR1 (Figure 12. step 2). This product was ligated with EcoR1 digested p18smahis with 0.5µl of Mfe1 (10U/µl) and 0.5µl of EcoR1 (10U/µl) in the ligation mixture. This prevented unwanted ligations (Figure 12. step 3) and improved the success rate of the desired ligation (Figure 12. step 4). DH5α cells transformed with ligation mixture were cultured on LB agar plates containing ampicillin at a concentration of 100µg/ml (see 3.3.4). Plasmids were screened using standard taq PCR and agarose gel electrophoresis, purified using qiagen miniprep kits and sequenced.

4.3 Results part 1: Vector development

Amplification of a pUC18 derivative using P18es2.6 (5'GGGGAATTCTGCGCG GAAC-3') and P18es800+ (5' GGGGAATTCGCAGGAAAGAACATG 3') produced a PCR product of 1.8Kb

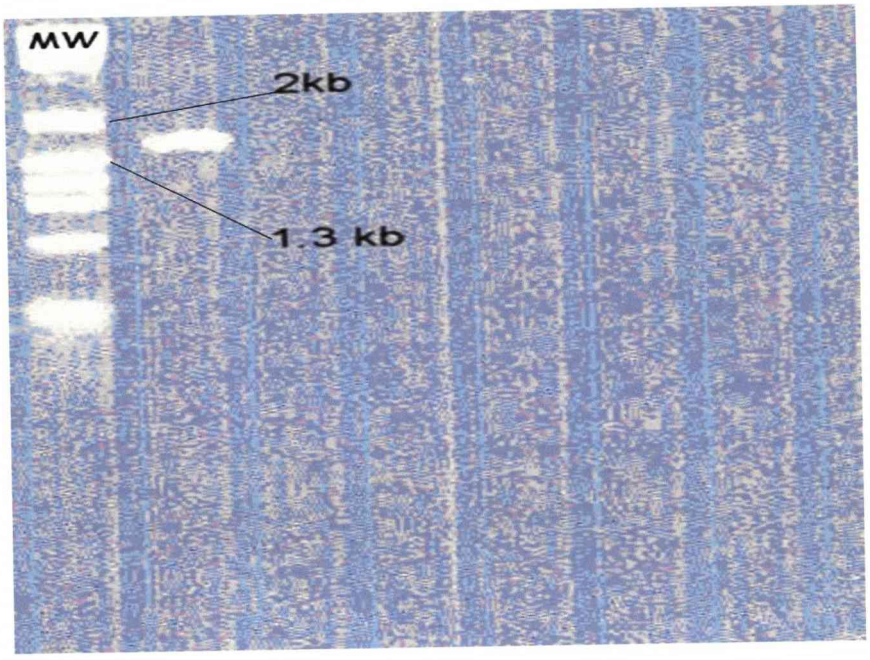


Figure 13 PCR product amplified from a pUC18 derivative. size ~1.8kb on an agarose gel, stained with ethidium bromide.

Bacterial colonies transformed with the above, were screened using primers that gave a product of 300 base pairs (bp). Figure 14 shows an example of two colonies that contained plasmid with the desired insert.

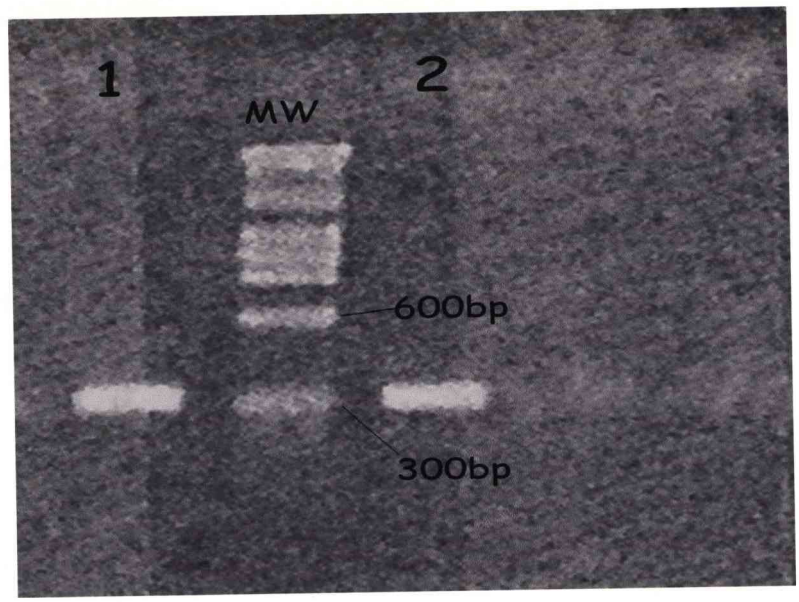


Figure 14. Example of two *E.coli* clones containing the vector shown in Figure 13. Identified via PCR screening. Product size ~300bp on an agarose gel, stained with ethidium bromide.

In total, five clones contain the vector. Two of these were shown to have the correct nucleotide sequence (Figure 15).

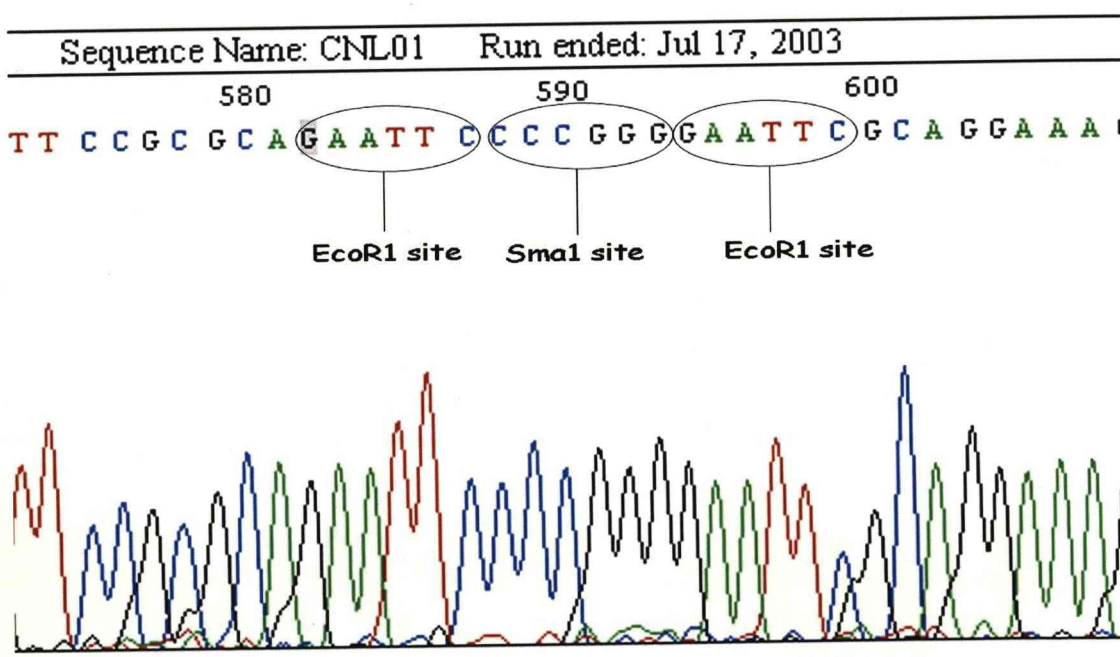


Figure 15. Sequence of introduced RE sites EcoR1 and Sma1 in p18smahis

These two vectors were used for the second stage of development in which a his tag stop region was added. Again colonies were screened using PCR. Primers were designed to produce products of 320bp as shown in 7 of the 25 samples screened in Figure 16.

Figure 16. Seven *E.coli* clones containing the desired vector P18sma-his (lanes 6, 8, 9, 14, 15, 19 and 22). Identified via PCR screening. Product size ~320bp on an agarose gel, stained with ethidium bromide

Sequence analysis of the above showed that five of the seven clones had the correct sequence (Figure 17).

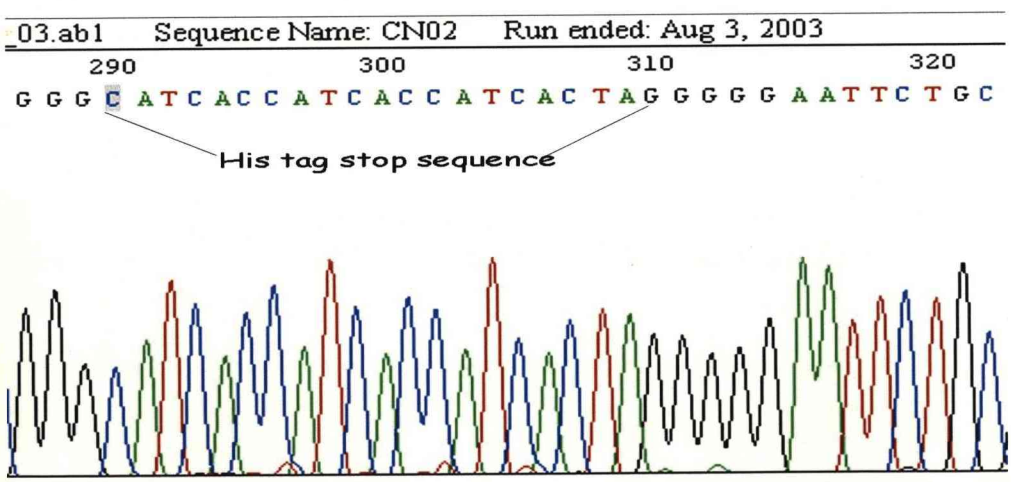


Figure 17. The his tag stop sequence in p18smahis.

Addition of the T7 termination sequence illustrated in Figure 12 involved several steps; Figure 18 shows two Mfe1 digests that extracted the T7 termination sequence from Ctpe and this was then ligated into EcoR1 digested p18smahis

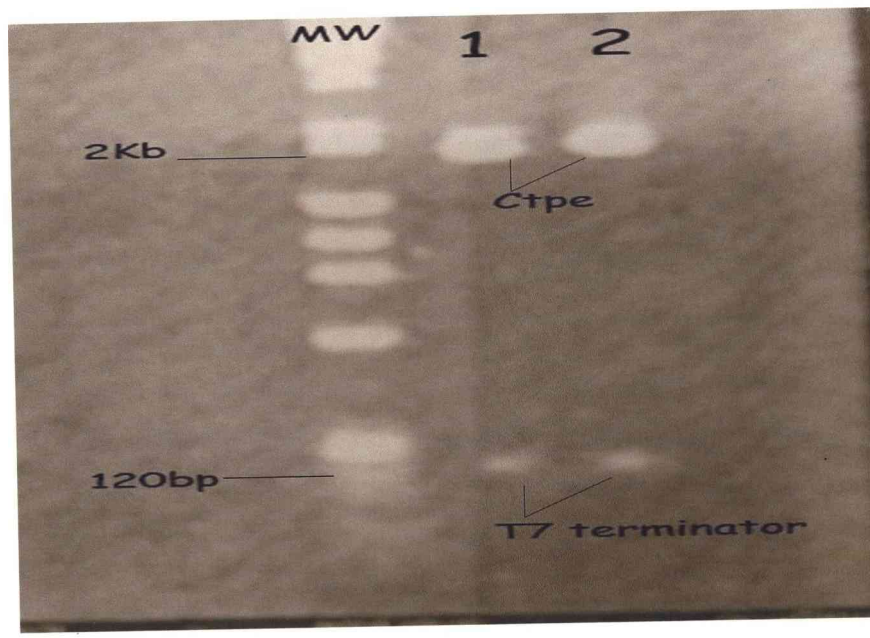


Figure 18. Two successful Mfe1 digests excising the T7 termination sequence from Ctpe. Product Sizes: T7 terminator ~150bp and Ctpe ~2Kb on an agarose gel, stained with ethidium bromide.

Colonies containing the insert were identified by PCR and the resulting DNA was sent for sequencing analysis. All samples had the correct, entire T7 termination sequence. The start of this sequence is shown in Figure 19.

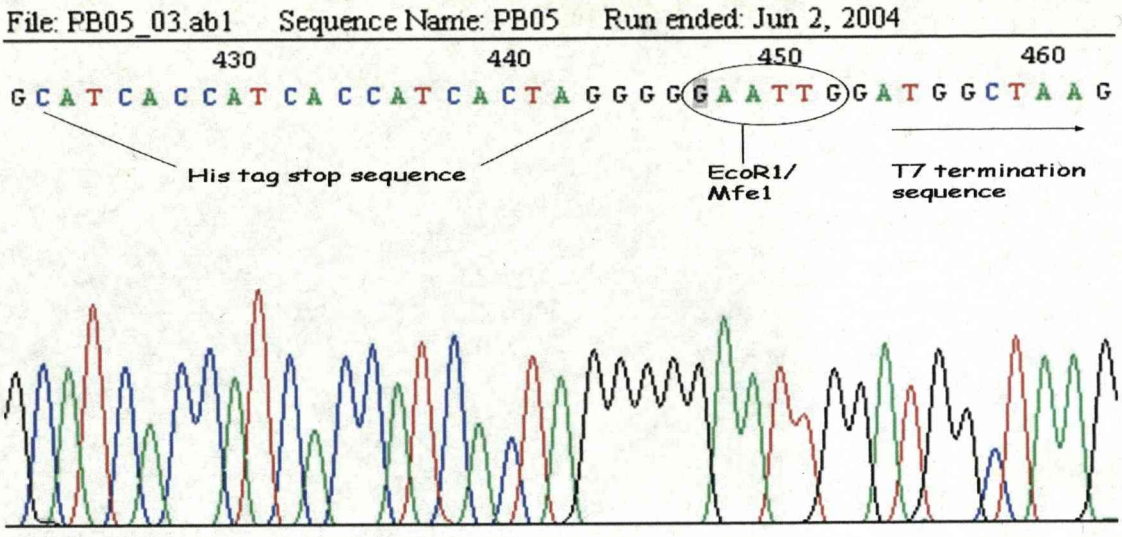


Figure 19. Addition of a T7 termination sequence to p18smahis: the combination of EcoR1 and Mfe1 restriction enzyme recognition sequences.

4.4 Materials and methods part 2: Amplification of APV genes and GFP: ligation with p18smahis

APV genes F, N, and P were amplified in a standard Pfu PCR from existing clones using the PCR primers listed below. Each oligo pair introduced a T7 promoter followed by a RBS sequence to the start of the gene, and removed the stop codon from the trailer.

Nucleocapsid primers

N-T7 **5'**-TTAATACGACTCACTATAGGTTAAGAAGGAGAGTCAAAAATGTCTCTTG-**3'**

with N-no-stop **5'**-CTCAAATTTGGATGATCTCTCATC- **3'**

Phosphoprotein primers

P-T7 **5'**-TTAATACGACTCACTATAGGTTAAGAAGGAGAAGTAACAATGTCTTTCC-**3'**

with P-no-stop **5'**-CAGATCAAGATTGTATATGTCGCTC **-3'**

Fusion protein primers

F-T7 **5'-**TTAATACGACTCACTATAGGTTAAGAAGGAGATATACATATGGATGTAAG

AATCTGT-**3'** with F-no-stop **5'**-ACTGACATAAGCCATGCTGCTATG-**3'**

GFP gene amplification

GFP was amplified from a plasmid kindly provided by Mathew Davis (University of Leeds) with primers T7-GFP

5'-TTAATACGACTCACTATAGGTTAAGAAGGAGACGCCACCATGGTGAGCAAGGGC-**3'** and GFP no stop **5'**- CTTGTACAGCTCGTCCATGCC **-3'**

4.4.1 Ligations with p18smahis, generating clones for expression

Blunt end PCR products were ligated into the Sma1 site of p18smahis with 0.5µl (10U/µl) of Sma1 in the ligation mixture. Ligations were used to transform DH5α competent cells which were grown on LB agar plates containing 100µg/ml of ampicillin. Subsequent colonies were prepared using the crude standard miniprep technique (section 3.3.5) for plasmid analysis. These were screened using EcoR1 restriction endonuclease digestion. Regions of interest were amplified using standard Taq PCR for sequencing.

4.5 Results part 2: APV and GFP gene amplification

Pfu PCR amplification of genes N, P, F and GFP produced blunt end products of 1.2Kb, 800bp, 1.6Kb and 700bp respectively as shown in Figures 21 and 22.

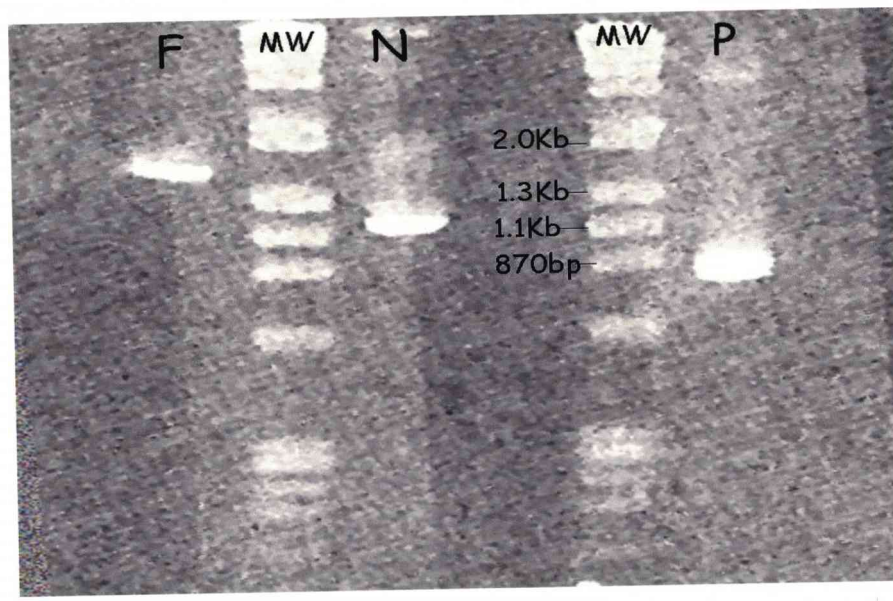


Figure 20. APV genes F, N and P (agarose gel, stained with ethidium bromide)

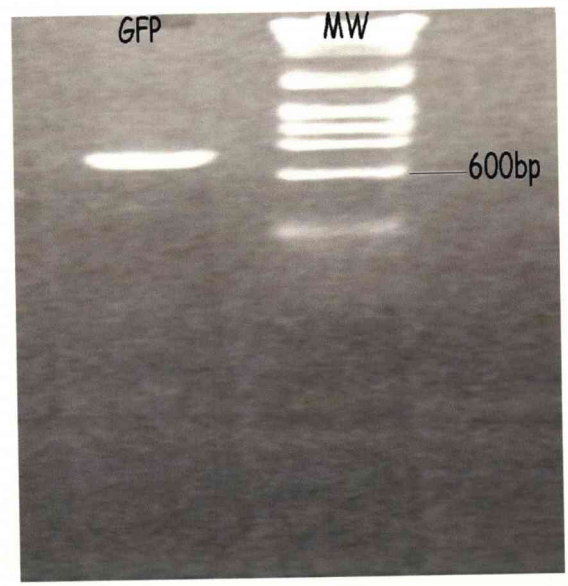


Figure 21. GFP gene (agarose gel, stained with ethidium bromide).

4.5.1 Plasmid analysis

EcoR1 digestion of p18smahis plasmids that had accepted the genes shown in Figures 21 and 22 resulted in excision of the insert producing the following sized products:

N-p18smahis = 1.8Kb + 1.2Kb

P-p18smahis = 1.8Kb + 800bp

F-p18sma-his = 1.8Kb + 1.6Kb

GFP-p18sma-his = 1.8Kb + 700bp

These are demonstrated in Figures (23, 24, 25 and 26)

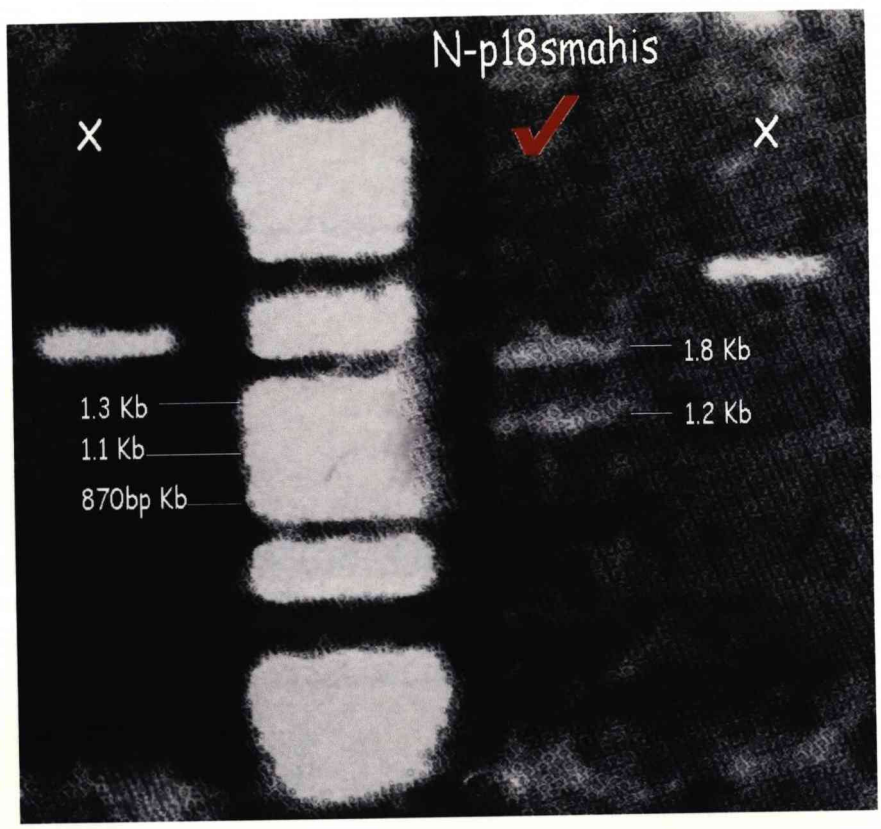


Figure 22. EcoR1 digest of three individual, N-p18smahis plasmid preparations. One produced the expected sizes of 1.8Kb and a 1.2Kb on an agar gel stained with ethidium bromide.

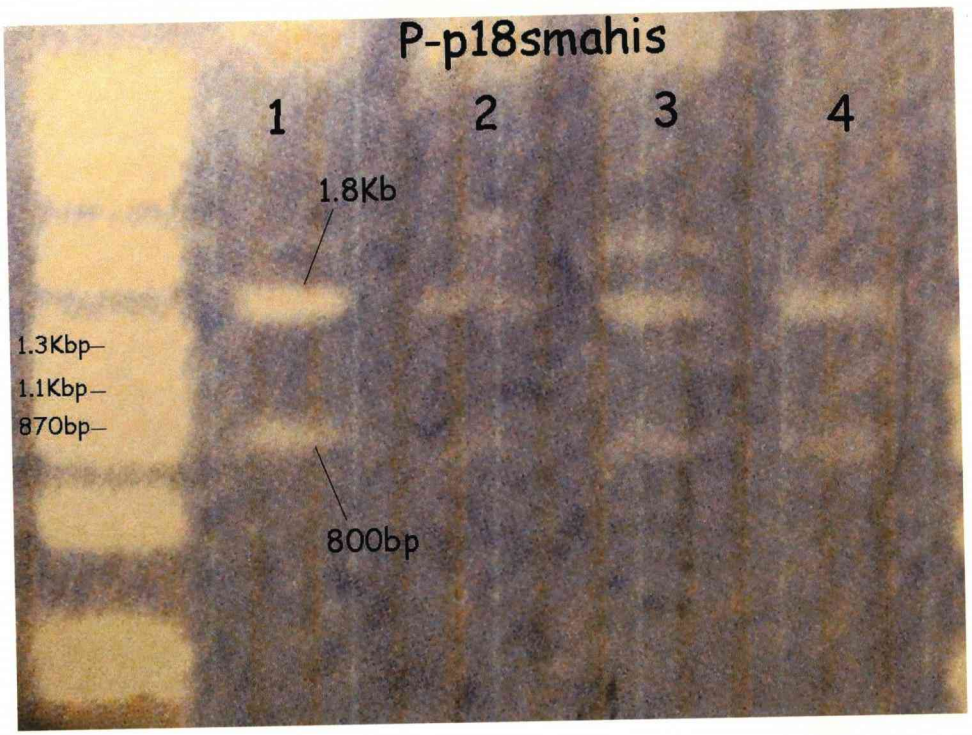


Figure 23. EcoR1 digest of four individual, P-p18smahis plasmid preparations. All produced the expected sizes of 1.8Kb and 800bp on an agar gel stained with ethidium bromide.

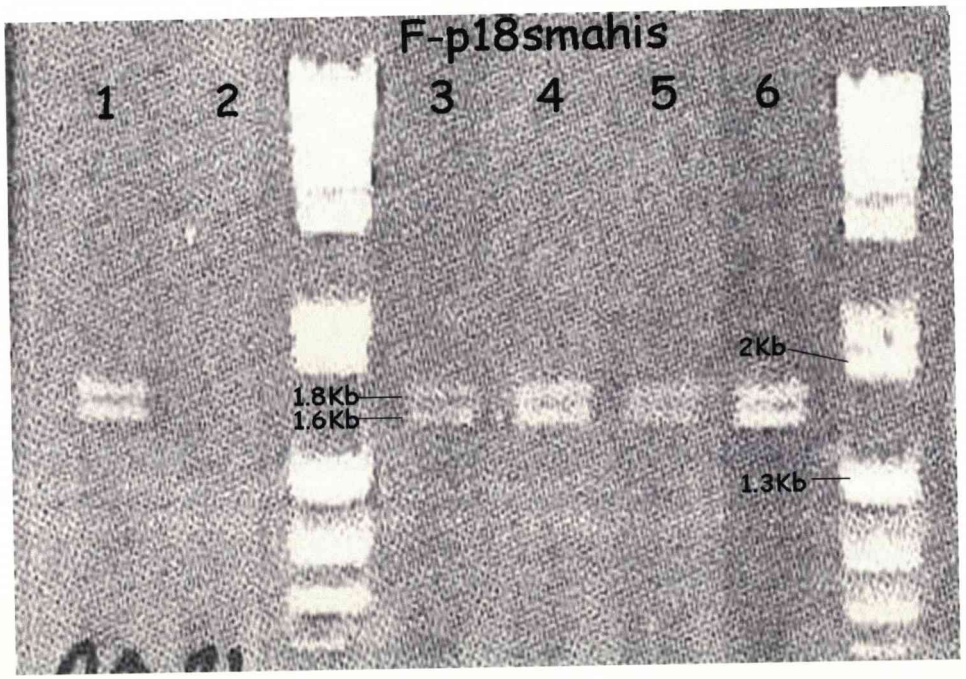


Figure 24. EcoR1 digest of six individual, F-p18smahis plasmid preparations. five produced the expected sizes of 1.8Kb and 1.6Kb on an agar gel stained with ethidium bromide.

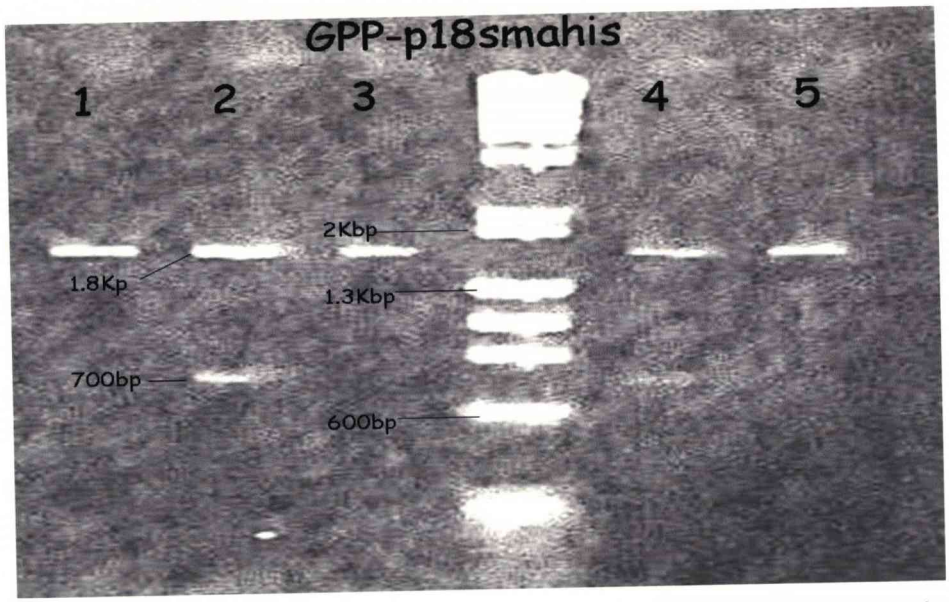


Figure 25. EcoR1 digest of five individual, GFP-p18smahis plasmid preparations. Two produced the expected sizes of 1.8Kb and 700bp on an agar gel stained with ethidium bromide.

4.5.2 Sequence analysis

Many sequences had two or more nucleic acid point mutations or deletions however; several clones of each construct had the correct sequence. Figure 26 shows the electroencephalogram of a GFP clone with the correct sequence.

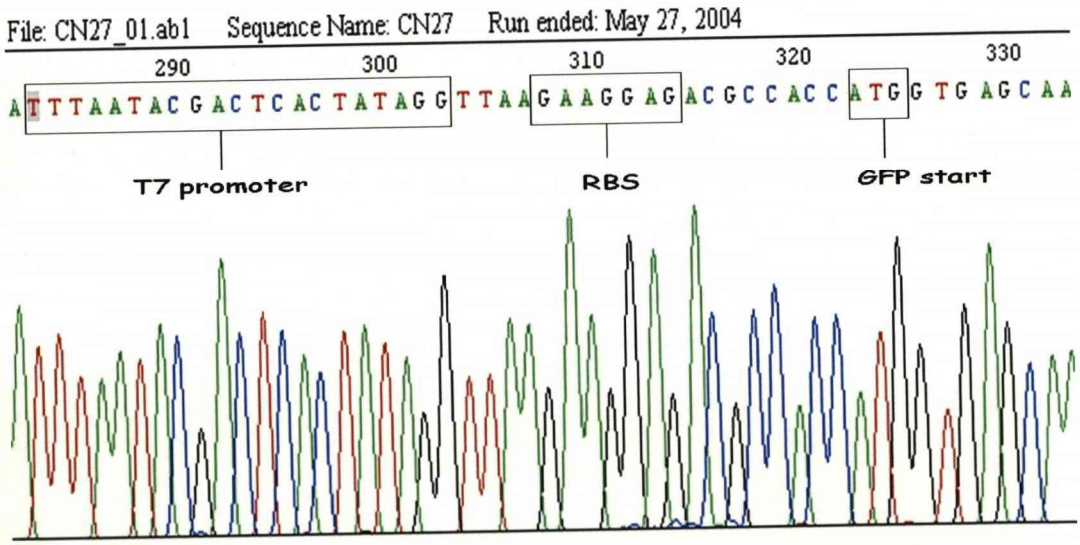


Figure 26. A GFP construct with an up stream T7 promoter and an RBS sequence.

4.6 Materials and methods part 3: Gene expression and purification

4.6.1 Transformations

E.coli BL21 (DE3) cells were transformed using ~1ng of N, P, F or GFP-p18smahis plasmid DNA according to the supplier's protocol (3.3.4)

4.6.2 PCR screening

Taq PCRs were used to screen BL21 (DE3) cells that had been transformed with N, P, F or GFP-p18smahis plasmid DNA. Colonies were screened directly from agar plates without the need for plasmid purification as shown in Figure 27. Products were analysed using agar gel electrophoresis (3.3.8)

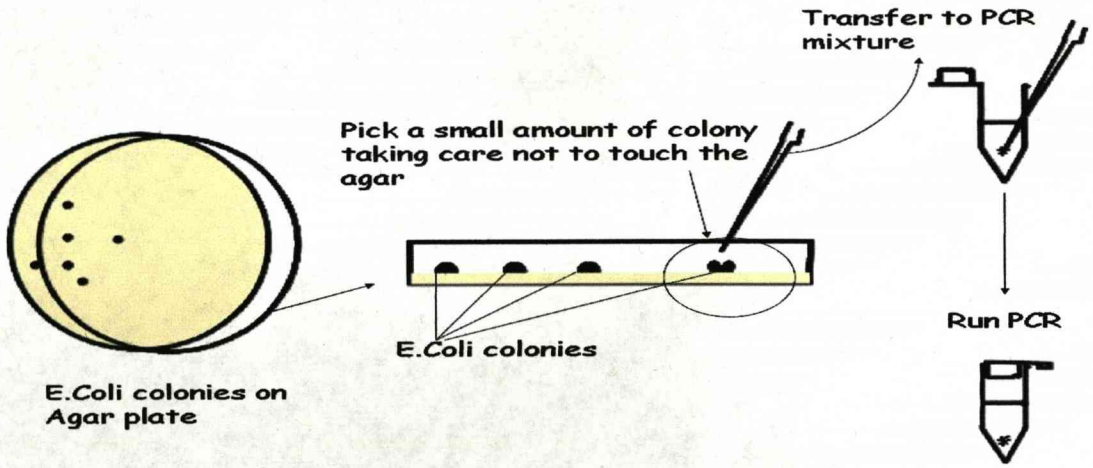


Figure 27. Direct PCR screening of *E.coli* colonies

4.6.3 Induction

Colonies that contained the desired plasmids were induced as described in section 3.4.1 using 1mM IPTG and an induction time of 3hours.

4.6.4 *E.coli* cell lysis

Cells were lysed as decribed in section 3.4.2

4.6.5 Protein purification

APV his tag recombinant proteins were purified under native conditions using Ni-NTA spin columns as described in section 3.4.3

4.6.6 Wet culture slides

0.5µl of induced and non-induced GFP cultures were placed onto 76 x 26 mm microscope slides, overlayed with cover slips then viewed using a UV microscope using a filter optimized for observing Fluorescein..

4.6.7 SDS-PAGE

APV recombinant proteins were analysed under none-reducing conditions as described in section 3.6

4.6.8 Western blot

APV western blots were used to detect recombinant proteins in cell lysates and purified samples as described under section 3.6.2

Positive and negative controls for these blots were prepared as follows:

Positive control

$1x10^7$ (75cm cell culture flask) of Vero cells containing APV

Cell sheet re-suspended in 2ml PBS

Two cycles of freeze thawing

Centrifuged for 1min at 13.2 x 1000rpm

Supernatant discarded

Pellet re-suspend in none reducing sample buffer (Appendix) for SDS-PAGE

Negative control

A standard *E.coli* cell culture was generated and lysed following the same procedures as described for those containing expression vectors. These were then prepared for SDS-PAGE (section 3.6).

4.7 Results part 3: Expression and purification

4.7.1 Direct PCR screening of *E.coli* colonies containing N, P or F-p18smahis

BL21 (DE3) cells that had been transformed with F-p18smahis, were screened with internal and external primer combinations (see Figure 28, 1. and 2.). Those transformed with P-p18smahis or N-p18smahis were screened using external primer combinations. For F-p18smahis these were anticipated to give 420bp products and 2Kb products respectively as shown In Figure 29.

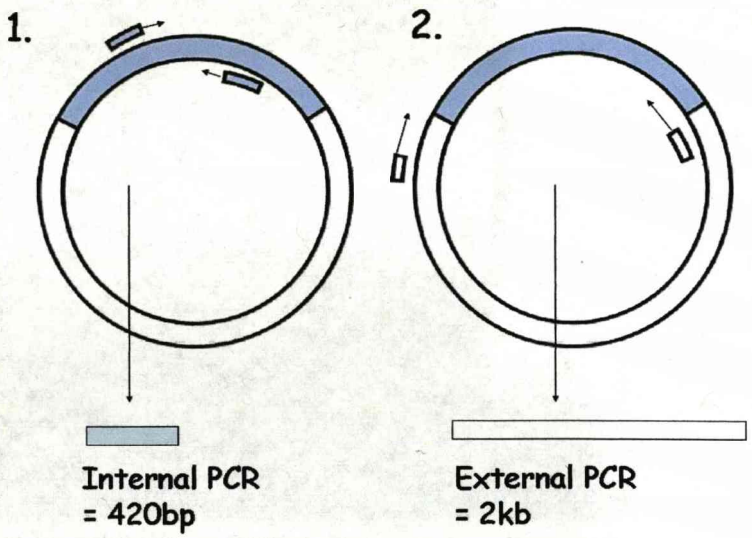


Figure 28. Internal and external PCRs for direct screening of F-p18smahis, P-p18smahis and N-p18smahis.

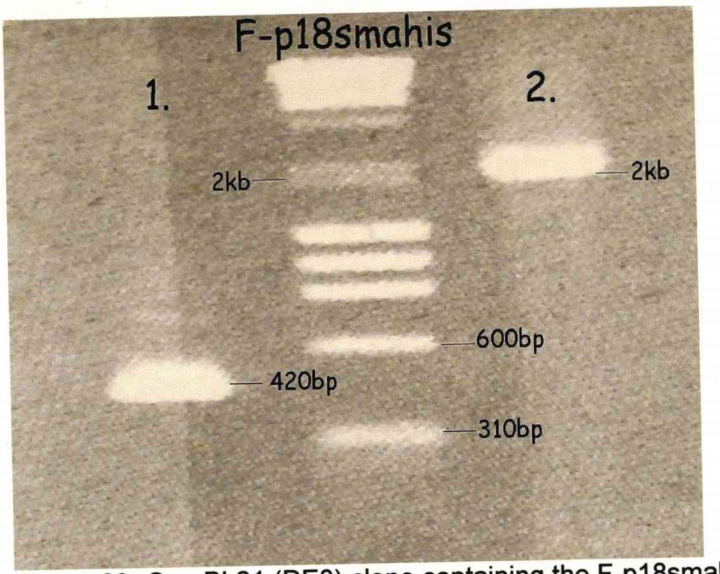


Figure 29. One BL21 (DE3) clone containing the F-p18smahis plasmid. 420bp and 2.0Kb PCR products on an agar gel stained with ethidium bromide.

Cells transformed with either N or P-p18smahis were screened using the external primer combination shown in Figure 28, 2, giving 1.8Kb and 1.4Kb products respectively as shown In Figures 30 and 31.

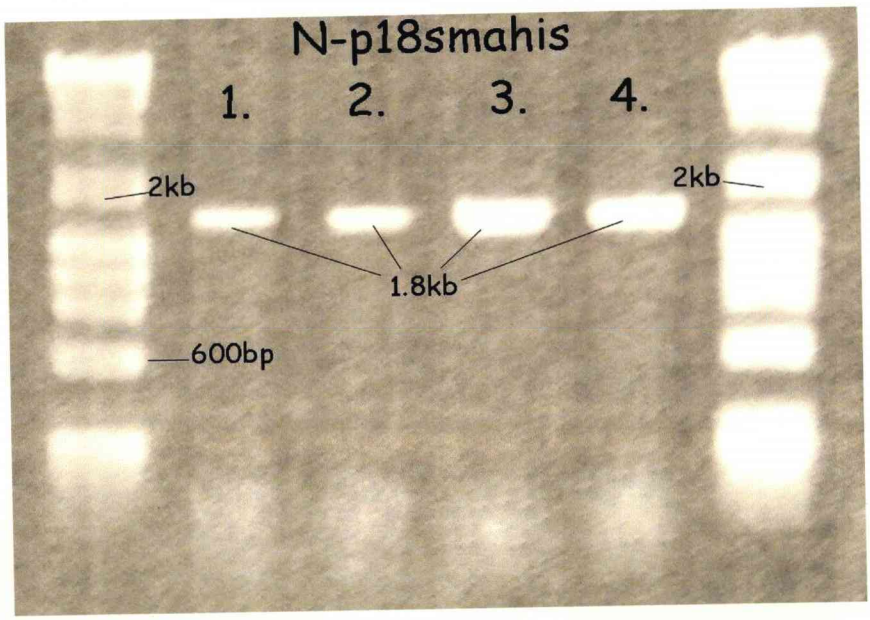


Figure 30. Four positive BL21 (DE3) clones containing the N-p18smahis plasmid. A 1.8Kb PCR product on an agarose gel stained with ethidium bromide

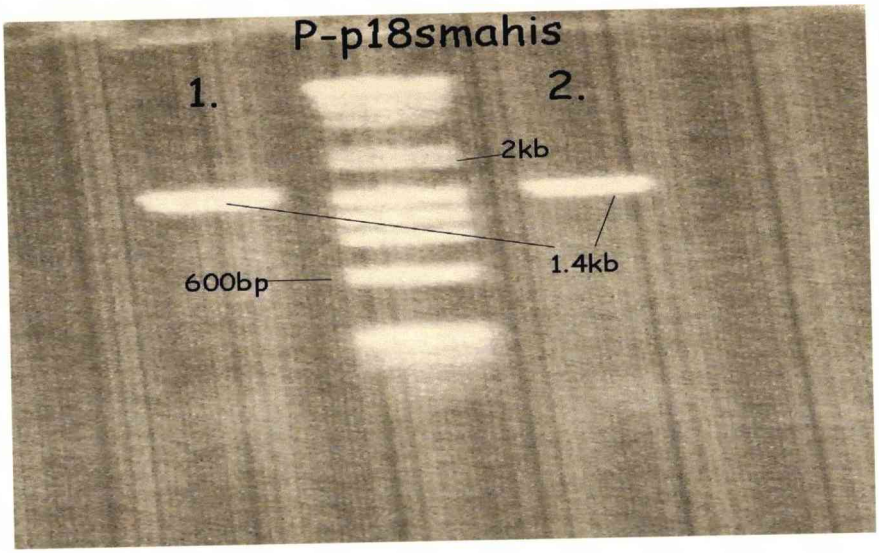


Figure 30. Two positive BL21 (DE3) clones containing the P-p18smahis plasmid. A 1.4Kb PCR product on an agarose gel stained with ethidium bromide.

4.7.2 GFP p18smahis expression

BL21 (DE3) cells that had been transformed with GFP-p18smahis were induced, prepared as wet culture slides and analysed directly by simple UV microscopy utilizing the inherent fluorescent properties of the GFP protein. Figure 31 shows induced *E.coli* cells (right) fluorescing compared with none-induced negative control (left). Thus: construct was functional and expression was induced.

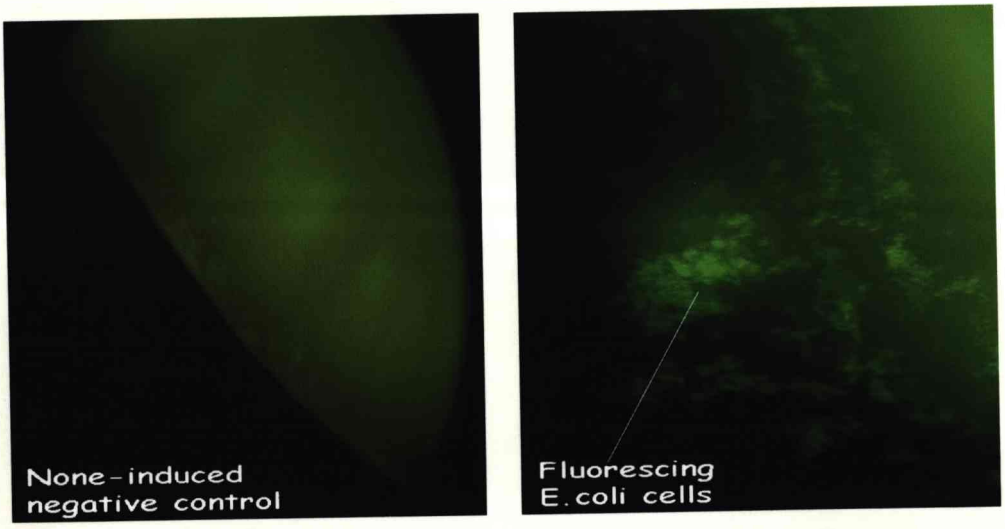


Figure 31. Wet culture slides of none-induced (left) and induced (right) *E.coli* cells containing GFP-P18sma-his construct viewed using UV microscopy.

4.7.3 Expression of APV constructs

APV constructs N-p18smahis, P-p18smahis and F-p18smahis employed the expression system validated by GFP above. None reducing SDS-PAGE was used for visualizing expression, comparing none-induced and induced samples. Expected sizes for the N and P proteins were as follows.

N = 38-43 KDa [14-16]

P = 35-40 KDa [14-16]

The F protein of APV has a cleavage site towards the amino terminal of the protein at an arginine-lysine rich sequence [48] and can be cleaved into two sub-units F2 (amino-terminal) and F1 (carboxy-terminal). In none-reducing conditions disulphide bonds link these sub-units but in reducing conditions the bonds are broken and the sub-units are separated. Therefore cleavage activity and conditions used in SDS-PAGE will determine the size of the product seen.

None cleaved F protein (F0) = 68 KDa [16]

Cleaved, none-reducing conditions = 59 KDa [16, 212]

Cleaved, reducing conditions F1 = 45-54 KDa F2 = 15 KDa [14-16].

F-p18smahis

The cell lysates of two F-p18smahis *E.coli* colonies (both induced and none induced) were compared using none reducing SDS-PAGE analysis. Figure 32 demonstrates bands (circled) in both induced samples which were absent in the none-induced samples indicating that these products were proteins resulting from induction i.e expression of F-P18sma-his. The approximate size of these products, 59 KDa, could represent sub-units F1 and F2, linked by disulphide bonds, as suggested by Cavanagh and Barrett 1988 [16] and Naylor [212].

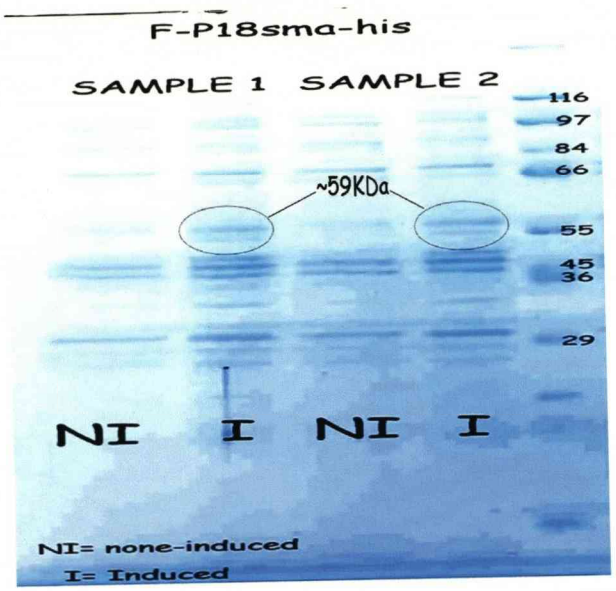


Figure 32 None-reducing, SDS-PAGE analysis of Cell lysates from two *E.coli* samples (Both, induced and none-induced) containing F-P18sma-his construct.

4.7.4 N and P-p18smahis

No differences were observed between the induced and none-induced cell lysates of these constructs via SDS-PAGE suggesting that expression had not been induced, however expressed N or P could have been masked by *E.coli* host proteins of the same molecular weight. Therefore, Ni-NTA purification using the his tag region was attempted.

4.7.5 Protein purification

Any expressed APV protein from p18smahis vectors would be fused with a his tag (Figure 12) which would allow purification using immobilized metal Ni^{2+}. Fractions that were taken at three separate stages of purification for F, N and P are shown in Figures 33 and 34. These were analysed by SDS-PAGE. The third fraction represents purified his tagged protein.

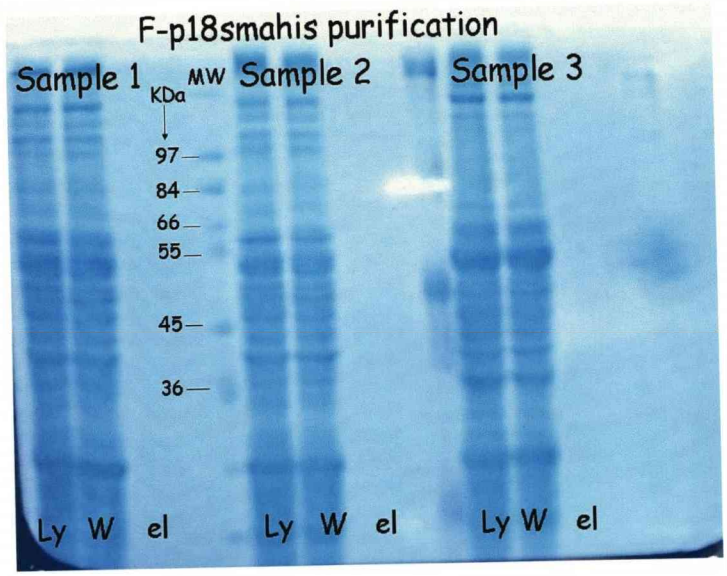


Figure 33 SDS-PAGE analysis of three induced and purified F-P18sma-his samples. **Ly** = cell lysate, **W** = wash fraction, **el** = purified sample.

No product could be seen in the purified sample for F in this gel or in other instances when IPTG concentration and/or time allowed following induction were altered.

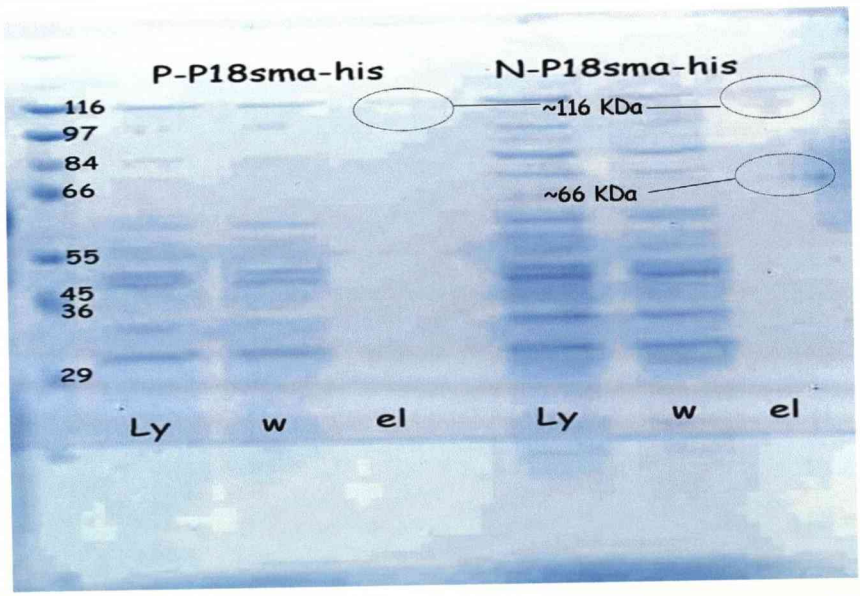


Figure 34. SDS-PAGE analysis of N and P-P18sma-his samples after induction and purification. **Ly** = cell lysate, **W** = wash fraction, **el** = purified sample. Ringed bands highlight faint bands seen in the purified sample.

Purified products of both N and P were larger than the expected sizes of P 35-40KDa, N 38-43Kda and most likely represented *E.coli* contaminants however, these could also have represented multimers (aggregates held together with none-covalent bonds) of each purified protein.

4.7.6 Western blot analysis

The lysates and purified samples of F, N and P (Figure 32, 33 and 34) together with APV virus culture and E Coli without insert were probed using polyclonal APV positive turkey serum to produce the blot shown in Figure 35.

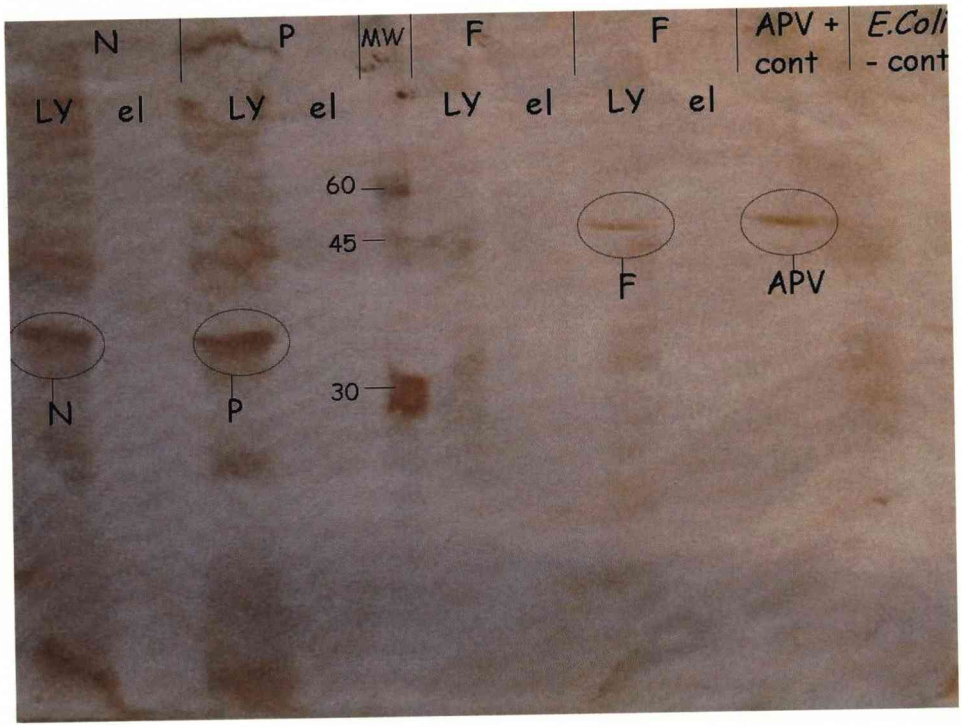


Figure 35. Western blot analysis of F, N and P recombinant proteins **Ly** = cell lysate and **el** = purified sample. Ringed bands highlight proteins that gave the strongest reaction

A graph plotting the relative frontal mobility (Rf) value of each MW marker (Table 1) against the $\log_{10}$ of its MW was used to determine the size of the bands highlighted in Figure 35. Rf values were calculated using:

Rf = Distance moved by sample (mm) / Distance moved by coomassie blue (mm)

Table 1

Rf values MW	Rf values ringed samples Figure 35	Log_{10} MW markers
0.018	N = 0.38	5.34
0.086	P = 0.38	5
0.164	F = 0.19	4.78
0.3	APV = 0.19	4.65
0.473		4.48
0.591		4.3
0.773		4.1
0.945		3.9

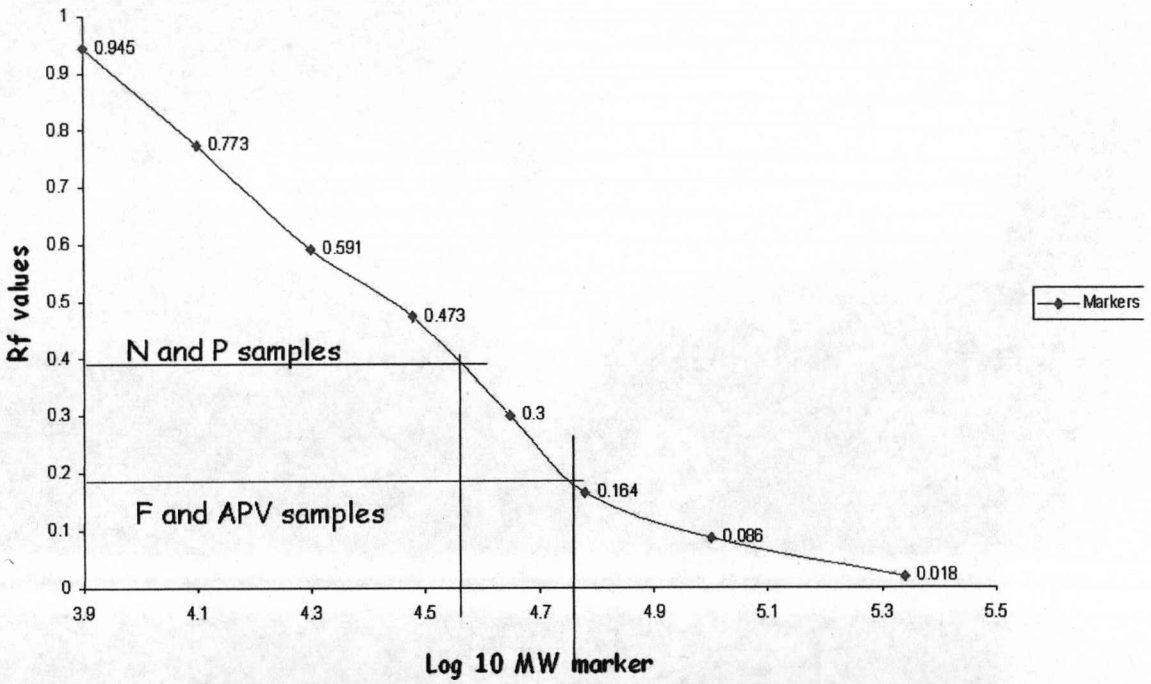


Figure 36. Log_{10} MW of samples N, P, F and APV following western blot analysis.

Western blot results and conclusions

Both the N and P lysate samples gave a 36.3KDa band. This size could have represented the P protein however; as both lysates produced the same size product, it was unlikely. Furthermore the purified samples of both N and P in Figure 34 did not show N or P bands and therefore they were considered to be *E.coli* proteins and not N or P multimers.

Both the F lysate and virus lysate gave a 57KDa band. This figure was close to the 59KDa published size for cleaved F protein [16, 212] in none reducing conditions. No products were seen in purified F samples.

It appeared from these results that the F protein was being expressed but not purified. One possibility may have been due to concealment of the his tag by the protein itself, which would render it inaccessible for capture, using Ni2+. A simple experiment to linearise the protein and expose the his tag regions before Ni2+ capture was attempted.

4.8 Linearising expressed protein

(i) Method

To disrupt hydrogen bonding and yield a linearised protein 8M urea was added to 200µl of the F cell lysates shown in Figures 32, 33 and 35. Mixtures were incubated for 1 hour and purified as described in section 3.4.2 under denaturing conditions.

(ii) Results

No F protein was purified from lysates treated with urea (Figure 37)

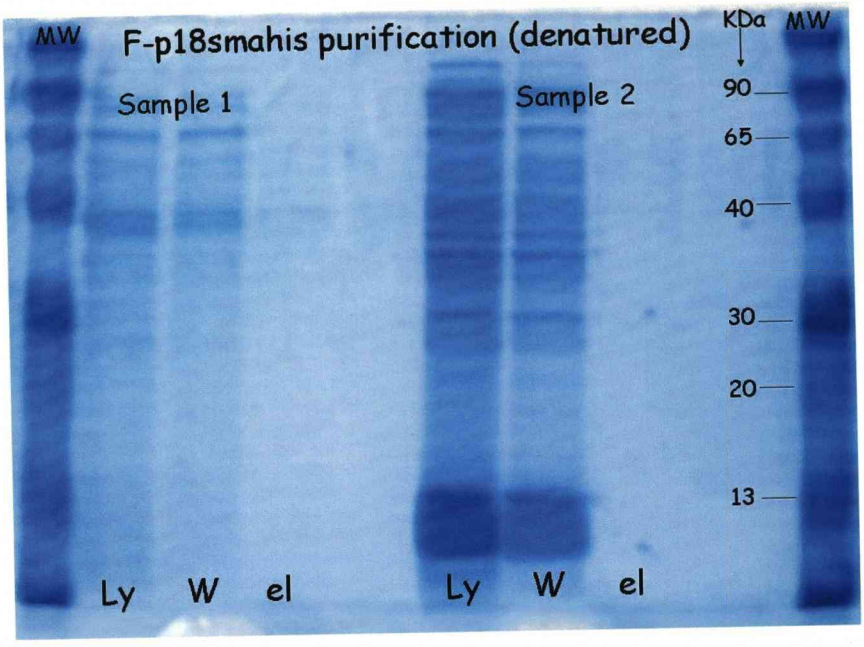


Figure 37. SDS-PAGE analysis of purified F protein following treatment with 8 M urea **Ly** = cell lysate, **W** = wash fraction, **el** = purified sample.

4.9 Discussion

Many proteins have been successfully expressed in and purified from *E.coli* cells, including glycoproteins of infectious laryngotracheitis virus (ILTV) [213] and APV N and M subtype C proteins [2, 3]. Here potentially antigenic subtype A APV proteins, the fusion protein (F), the nucleocapsid protein (N) and the phosphoprotein (P) were tagged with 6 histidine amino acids and selected for expression in *E.coli*. In addition to these proteins GFP was expressed as a direct visual reporter of gene expression and purification.

A plasmid, known to readily accept APV genes was modified so that it could be used as a prokaryotic expression vector. Although many commercial plasmids are available for expression, certain APV genes were known to be difficult to clone (C. J. Naylor., D. R. Kapczynski., personal communication); therefore, the rational was to make necessary changes to a plasmid with known cloning characteristics. The methods adopted for making these changes were effective and generated some flexible cloning strategies that proved useful in future work. In particular the strategy outlined in Figure 12 produced a method that was highly selective for desired ligations. The T7 terminator was added by ligation of 2 dissimilar RE sites in the presence of both restriction endonucleases as previously described. This proved to be an efficient method whereby 90 % of screened colonies contained the desired insert.

Figures 22-25 demonstrated p18smahis plasmids that had accepted the genes N, P, F and GFP although the plasmid yields were low. As a convenient alternative to further bacterial culture and DNA purification when the inserted genes were sequenced, plasmid DNA was amplified by PCR. Only a small number of these

clones contained the correct sequence, emphasizing the importance of sequencing the constructs, in their entirety, before taking them onto the next stage. For a gene to be expressed it must be "in frame" (multiples of three nucleotide bases from the start codon to the stop codon), therefore, just one base deletion will eliminate desired protein expression beyond that point in the gene.

The GFP was useful for readily determining protein expression and showing that the developed vector was functional. While expression generally increases over time, over expression can lead to accumulation of insoluble protein in inclusion bodies. When bacteria package highly expressed protein in this manner, strong denaturants are required for their extraction. Unfortunately such strong denaturants can affect a proteins antigenic property; therefore if a protein is to be used as antigen it is desirable to extract it in soluble form. Optimal conditions for this can vary from protein to protein and are often achieved empirically.

The cell lysates of induced *E.coli* containing the F gene (Figure 32) showed products of 57KDa following western blot analysis as did the APV virus culture. This was close to the published size 59KDa [16] and may have represented sub-units F1 and F2, linked by disulphide bonds, however IMAC Ni-NTA purification yielded no protein. This may have been because too little was being expressed to allow purification but another possibility was concealment of the his-tag within the protein itself, which would render it inaccessible for capture, using Ni^{2+}. However, by adding a strong denaturant to the cell lysate, hence linearising at least a percentage of the protein, the His-tag should have been exposed and accessible for purification. These changes had no impact on purification efficiency. As consistent bands of the correct size were seen in cell lysates of

induced F samples, it is likely that expression levels were insufficient for purification.

Purification from N and P lysates yielded several products, yet these were larger than expected (Figure 34). It was thought that these could have represented N and P multimers yet, none of these proteins reacted in a western blot when probed using an APV positive serum. This suggests that the proteins observed by SDS-PAGE were *E.coli* contaminants purified non-specifically on the Ni-NTA columns.

Generally, a low level of expression was considered to be the reason for the inability to purify the proteins and this led to exploration of methods that increased expression levels.

It is known that different RBS and their position relative to the to the downstream transcribed gene can affect translation, as can other factors within the plasmid vector [214, 215]. However, to introduce these changes to existing clones would be time consuming and may only improve expression levels minimally. An alternative approach was to use a commercial vector. In addition it was decided that fusion proteins of APV and GFP would be made to enable simple UV visualization of APV protein expression and tracking of the different stages of purification. A baculovirus expression system was also explored.

Chapter 5

Expression using commercial *E.coli* and Baculovirus vectors

Contents **Page**

Chapter 5

Expression using commercial *E.coli* and Baculovirus vectors

5.1 Introduction

(i) *E.coli*

GFP and APV N, P and F genes were cloned into a commercial, prokaryotic expression vector p-ET 30 (kindly provided by Dr Richard Birtles, Liverpool University). This vector is shown in Figure 38.

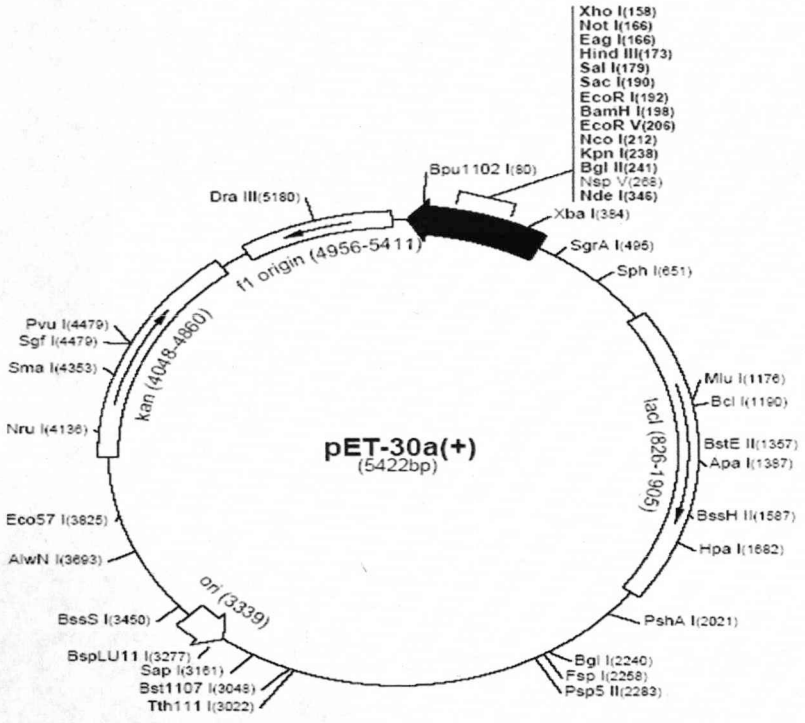


Figure 38. Pet_{30} vector map

Like p18smahis it placed genes under the control of a T7 promoter and had the potential to provide either or both amino (N) and carboxy (COOH) terminal his tagged proteins following expression. N-terminal his tags were generated for the N and P proteins and dual N / COOH terminal his tags for the F protein. In addition, APV genes N, P and F were each combined with the GFP gene to

produce new N-GFP, P-GFP and F-GFP proteins, when expressed (Figure 39). For each of the 3 fusion constructs, single and duel his tags were made. The GFP region of these new recombinant APV proteins enabled tracking of expression and purification. It was important to place the GFP gene after AVP genes so that expressed, fluorescing protein could be attributed to APV-GFP and not GFP alone.

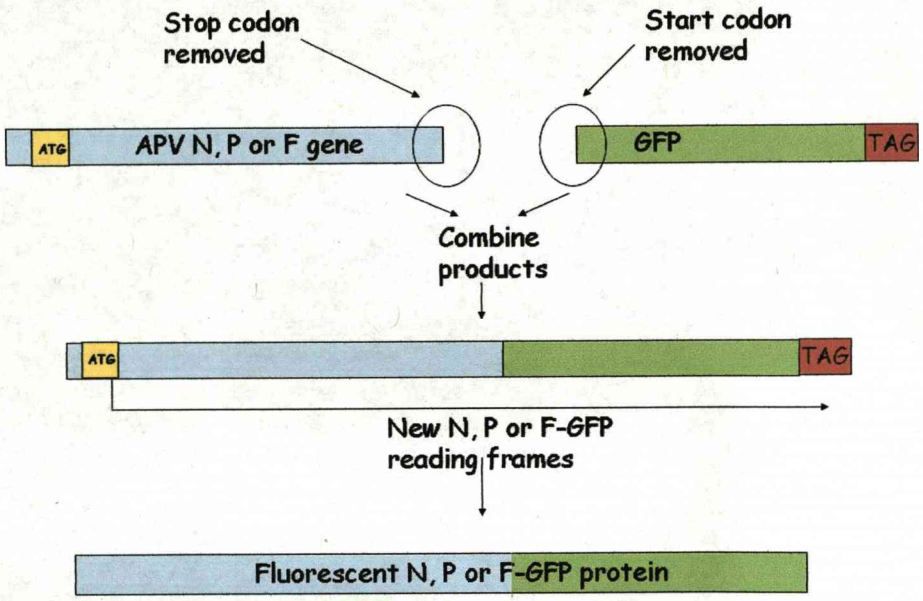


Figure 39. Generating F-GFP recombinant proteins.

(ii) Baculovirus

Baculoviruses have been used to express many different viral proteins in insect cells [1, 4-6, 63]. These systems have been shown to produce high levels of correctly folded recombinant proteins when expressed under the control of the *Autographa californica* multiple nuclear polyhedrosis virus (AcMNPV) polyhederin promoter [216-219]. Insect cells recognise signals for phosphorylation and N-linked and O-linked glycosylation amongst others and are therefore capable of

producing biologically active proteins. As the APV F protein is known to be one of the major immunogenic, glycosylated proteins it was selected over N and P to be expressed in this system along with GFP. These genes were inserted into baculovirus transfer vectors for transfection of Sf9 insect cells with linear baculovirus DNA. Expressed recombinant proteins would have an N terminal his tag.

5.2 Materials and methods part 1: Developing clones

5.2.1 Amplification of APV, GFP and APV-GFP genes for *E.coli* expression

N, P, F and GFP genes were initially designed to produce an N-terminal his tagged protein following expression from p-ET 30. Genes required amplification using oligo pairs that started in frame with the gene start codon and finished at the translational stop codon. (Figure 40)

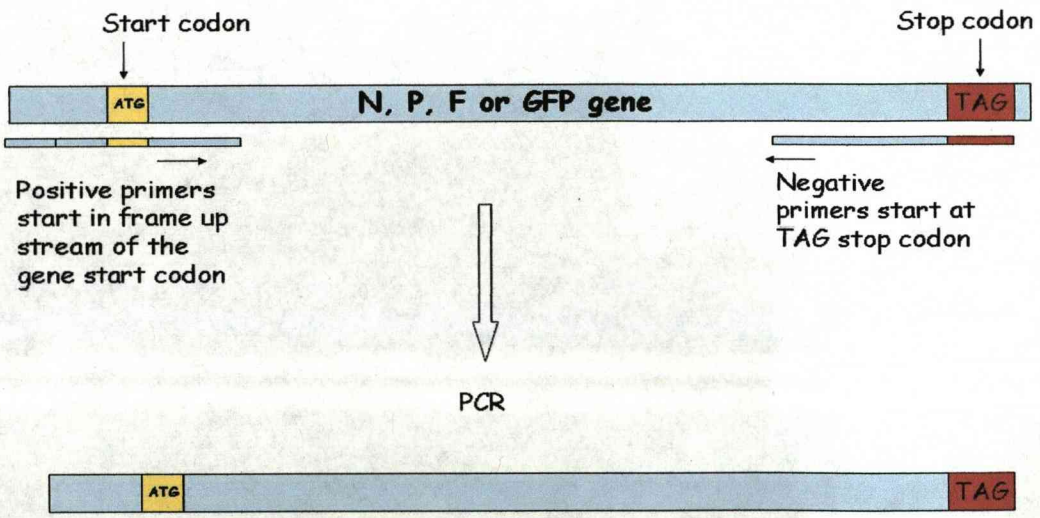


Figure 40. Generating new N, P, F and GFP genes for ligation with Pet30

Several templates were used for amplifying APV genes: (i) The full length clone developed by Naylor et al 2004 [72] using oligos listed in Table 2 (ii), existing N and P clones in PCi (Promega, UK) using N and P oligos listed in Table 2 and (iii) the F-p18smahis clone developed in Chapter 4 using oligos listed in Table 3. Use of this clone for amplification of the F gene produced a gene that coded for both N and COOH terminal his tags.

Table 2

Gene generated Oligo Names	**Sequence 5' to 3'**
N	
N start plus 3	AAAATGTCTCTTGAAAGTATTAG
Pstart neg	CAGGGAAAGACATTGTTAC
P	
Pstart pos	GTAACAATGTCTTTCCCTG
Pstop	CATAACTACAGATCAAGATTG
F	
Fstart plus 3	AGGATGGATGTAAGAATCTGTCTCC
LTZ 4.6 neg	CATATCTGCAGGGATTTCGCCTAGACATCTTC

Table 3

Gene generated Oligo Names	**Sequence 5' to 3'**
F	
Fstart plus 3	AGGATGGATGTAAGAATCTGTCTCC
Sma His neg	CTAGTGATGGTGATGGTGATGCCC

The GFP gene was amplified from GFP-p18smahis developed in Chapter 4 using oligos listed in Table 4.

Table 4

Gene generated Oligo Names	**Sequence 5' to 3'**
GFP	
GFPstart 2	ATGGTGAGCAAGGGCGAGGAGC
GFP stop 1	TTTACTTGTACAGCTCGTCC

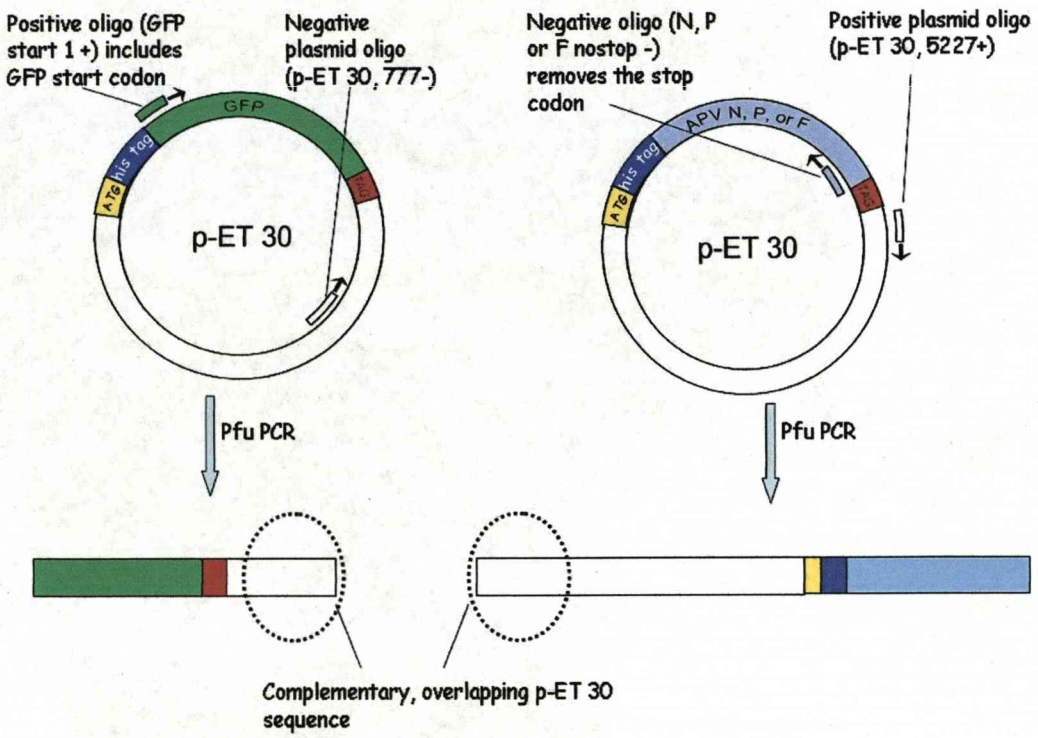
APV-GFP fusion genes were developed as illustrated in steps 1-4, Figure 41, using oligos listed in Table 5. However, these products were only developed after the GFP gene and each APV gene had been individually cloned into p-ET 30. This PCR product was circularized (step 5 Figure 41) producing a viable plasmid vector and therefore did not require further development.

All PCRs followed the standard Pfu PCR protocol. However the overlap PCR in step 3 Figure 41 had no oligos in the reaction mixture.

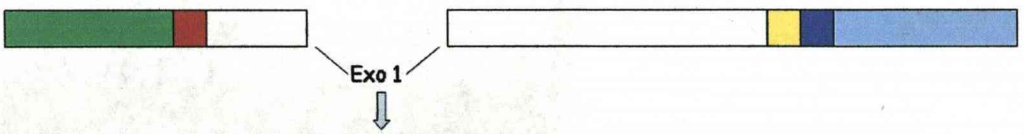
Table 5.

Oligo Names	**Sequence 5' to 3'**
GFPstart 1	ATGGTGAGCAAGGGCGAGG
p-ET 30, 777 –	CAGTCGTCACTCATGGTGATTTCTC
p-ET 30, 5227 +	GAGCTCCGTCGAC
N nostop	CTCAAATTTGGATGATCTCTCATC
P nostop	CAGATCAAGATTGTATATGTCGCTC
Fnostop	ACTGACATAAGCCATGCTGCTATG

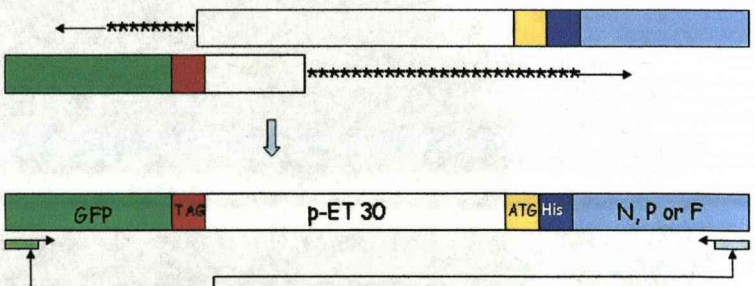
1. The GFP and APV genes were amplified from p-ET 30 constructs producing PCR products with regions of complementary, overlapping sequence



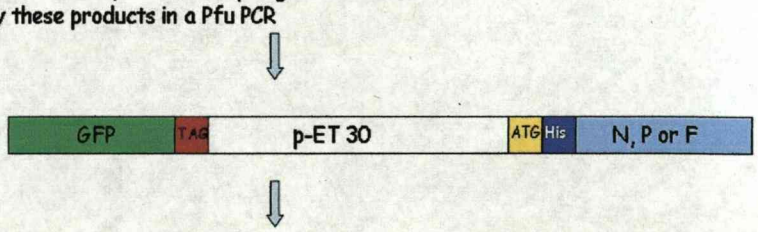
2. These were treated with exo 1 (Exo Appendix) to remove oligos: GFP start 1 +, p-ET 30, 777-, p-ET 30, 5227+ and N, P or F nostop -



3. PCR products were mixed and run in an overlap Pfu PCR



4. GFP start 1. and N, P or F nostop oligos were added to amplify these products in a Pfu PCR



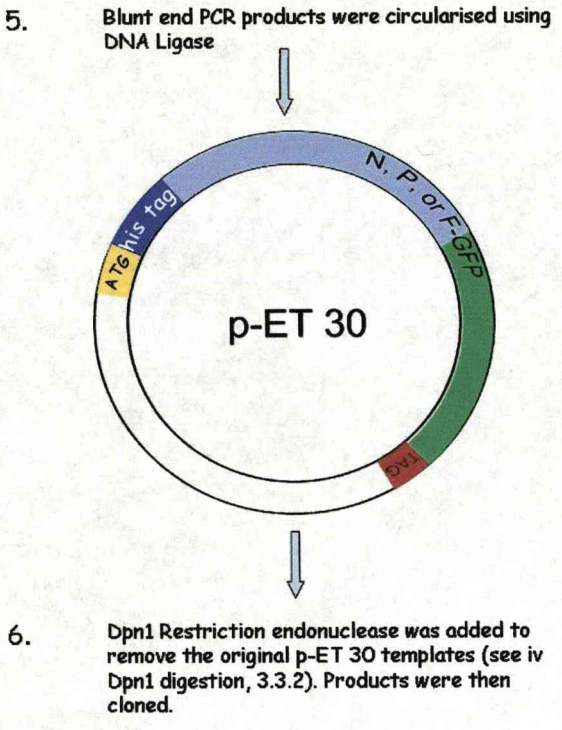


Figure 41. The generation of p-ET 30 plasmid vectors carrying APV-GFP genes

Vectors that coded for both N and COOH his tagged N and F-GFP proteins were also developed using the above vectors as templates in a SDM reaction (3.3.2). Oligos for these reactions are listed in Table 6

Table 6.

Oligo Name	**Sequence 5' to 3'**
SDMgfp-cterm-his-pos	CGAGCTGTACAAGAAATCGGATCCGAATTCG
SDMgfp-cterm-his-neg	CGAATTCGGATCCGATTTCTTGTACAGCTCG

5.2.2 Amplification of the APV F gene and the GFP gene for Baculovirus expression

Both genes were amplified in a standard Pfu PCR from F and GFP-p18smahis vectors developed in Chapter 4 using oligos that introduced Mfe 1 RE recognition sites to both the 5' and 3' ends. The sequence of these oligos is shown in Table 7. These sites were ultimately used for ligation with EcoR1 cut pBlueBacHis2 vectors (5.2.4).

Table 7.

Gene generated Oligo Names	**Sequence 5' to 3'**
Fmfe1pos	CGTTCCAATTGGATGTAAGAATCTGTCTCCTATTG
Fmfe1 neg	CAAGCCAATTGCAGACTGACATAAGCCATGCTGC
GFP mfe1 pos	CTTATCAATTGGTGAGCAAGGGCGAGGAGCTG
GFPmfe1neg	ATAGGCAATTGCGACTTGTACAGCTCGTCCATGCCG

5.2.3 *E.coli* and Baculovirus transfer vectors: Ligations and cloning

Blunt ended GFP, N, P and F PCR products were ligated into the EcoRV site of p-ET 30 using the Fermentas protocol but with the addition of 0.5µl (10U/µl) of EcoRV in the ligation mixture. Ligations were used to transform DH5α competent cells which were grown on LB agar plates containing 15µg/ml of Kanamycin.

Blunt ended N, P and F-GFP PCR products were circularized using T4 DNA ligase according to the recommended protocol. After an overnight incubation at

14°C, 0.5µl (2-8U/µl) of RE Dpn1 was added. This mixture was incubated for a period of 6 hours at 37°C then used to transform DH5α competent cells. These were again grown on LB agar plates containing 15µg/ml of Kanamycin.

Ligation of F and GFP genes into pBlueBacHis2 followed procedures used for inserting the T7 termination sequence into p18smahis (4.2). F and GFP replaced the T7 terminator and pBlueBacHis2 plasmids replaced p18smahis in the strategy illustrated in Figure 12. Ligations with pBlueBacHis2 were in the presence of EcoR1 only but all other constituents were the same. However, the APV F gene contained two internal Mfe 1 sites in addition to those introduced to the start and end of the gene (5.2.2) and therefore when excised from Ctpe using RE Mfe1, was digested into three sections, (Figure 42) each of which having complementary end sequences to that of EcoR1 digested pBlueBacHis2. GFP and all three sections of the F gene were ligated with EcoR1 digested pBlueBacHis2 with 0.5µl of EcoR1 (10U/µl) in the ligation mixture. DH5α cells transformed with ligation mixture were cultured on LB agar plates containing ampicillin at a concentration of 100µg/ml (3.3.4).

5.2.4 RE digestions

Digestions using EcoRV, EcoR1, Mfe 1 and Dpn 1 followed suppliers protocols.

5.2.5 Screening and sequencing

Restriction endonuclease (3.3.6) and PCR techniques (3.3.1 and 4.6.2) were used to screen *E.coli* colonies for desired plasmids. All products were analysed using agar gel electrophoresis. Those that were positive were sequenced.

5.2.6 Plasmid preparation

Qiagen miniprep kits were used for all plasmid preparations (3.3.5)

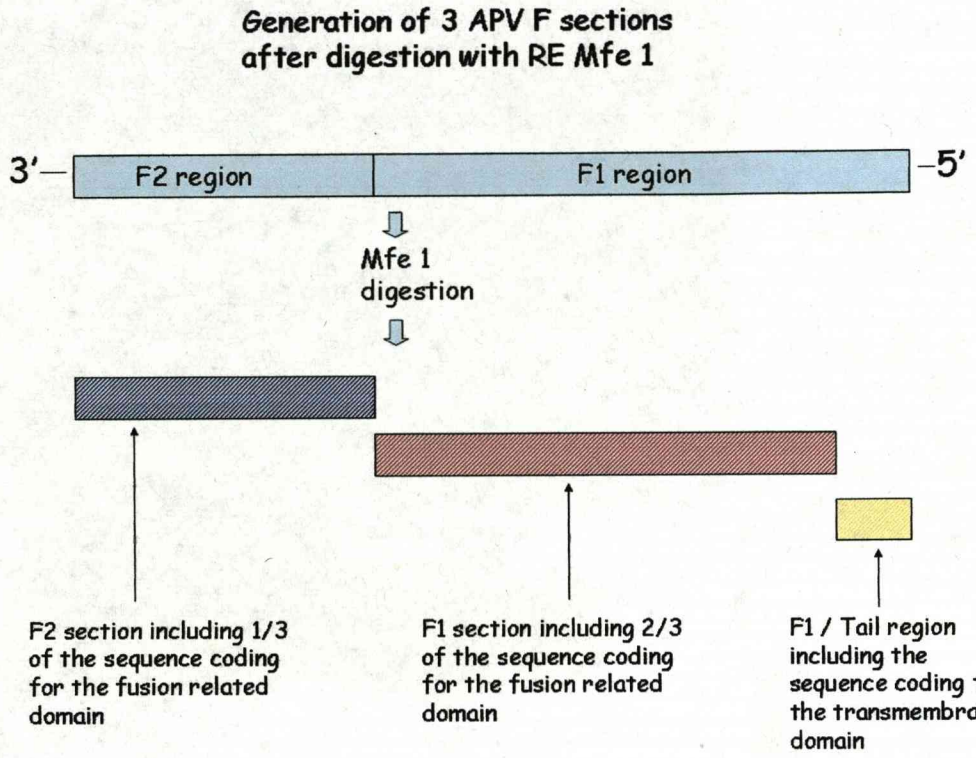


Figure 42. The F gene digested into three sections using RE Mfe1. Each section had Mfe1 5' AATT overhangs, complementary to EcoR1 5' AATT overhangs of pBlueBacHis2.

5.3 Materials and methods part 2: Expression in *E.coli*

5.3.1 Transformations

E.coli BL21 (DE3), BL21 (DE3) pLysS and BL21 (DE3) pLysE cells were transformed using ~1ng of N, P, F, GFP, N-GFP or F-GFP p-ET 30 plasmid DNA according to the supplier's protocol (3.3.4). pLysS and pLysE cells contain plasmids that express T7 lysosyme (more so in pLysE than pLysS). This reduces

expression of T7 driven genes until induced and can be useful if proteins are suspected of being toxic to bacteria.

5.3.2 PCR screening

Taq PCRs were used to screen BL21 (DE3) cells that had been transformed with the above. Colonies were screened directly from agar plates as described in section 4.6.2 and Figure 27.

5.3.3 Induction

Colonies that contained desired plasmids were induced as described in section 3.4.1 using either or both 0.5mM and1mM IPTG and induction times of 1-4hours.

5.3.4 *E.coli* cell lysis

Cells were lysed as described in section 3.4.2

5.3.5 Protein purification

APV his tag recombinant proteins were generally purified under standard native and denaturing conditions using Ni-NTA spin columns as described in section 3.4.3. In addition to the standard purification protocols the following modifications were made : 1. Addition of 2-Mercaptoethanol (2ME) (10mM final concentration) to denatured lysates. 2. Addition of 10% ethanol in denaturing wash buffers. 3. Addition of 25% glycerol in denaturing wash buffers and 4. Addition of 8M urea to native lysates and wash and elution buffers.

5.3.6 Wet culture slides

0.5µl of induced GFP and N, P and F-GFP cultures were placed onto 76 x 26 mm microscope slides, overlayed with cover slips then viewed using a UV microscope using a filter optimized for observing Fluorescein.

5.3.7 SDS-PAGE

All recombinant proteins were analysed under none-reducing conditions as described in section 3.6

5.3.8 Western blot

APV and his tag western blots were used to detect recombinant proteins in cell lysates and purified samples as described under section 3.6.2

5.3.9 ELISAs

Flexible, 96 well, flat bottomed ELISA plates (Becton Dickinson, UK) were used. Wells were coated with 50µl of antigen which had been previously diluted 1/50 in ELISA CBC (carbonate-bicarbonate) buffer (Appendix). Plates were incubated at 8^0C overnight. Antigens included cell lysates, purified products and a Liverpool developed whole virus APV antigen. The details of where each antigen was placed in a particular ELISA, is given along side the relevant plates in the results section.

The procedures for APV and his tag ELISAs are given below.

APV ELISA

1. Incubated plates washed 5 times with ELISA wash buffer (Appendix).
2. 50µl / well of polyclonal APV positive or negative serum, diluted 1/100 in wash buffer incubated for 1 hour at 37^0C.
3. Washed 5 times with wash buffer.
4. 50µl / well of GAT conjugate diluted 1/1000 in wash buffer incubated for 1 hour at 37^0C.
5. Washed 5 times with wash buffer.
6. 100µl / well of OPD activated with 30% H_2O_2 (Sigma, UK H-1009) (Appendix) incubated at room temperature in the dark for a maximum of 15 minutes.
7. 50µl / well of 2.5M H_2SO_4 (Appendix)

his tag ELISAs

1. Same as step 1 above
2. Monoclonal, anti his, HRP conjugated antibody diluted 1/10,000 in PBS/Tween for 1 hour.
3. Same as steps 5-7 above.

5.4 Materials and methods part 3: Baculovirus expression

5.4.1 Insect cell lines

Sf9 insect cells were grown and maintained as described in 3.5.2

5.4.2 Transfection

GFP and F2-BlueBacHis2 constructs were transfected as described in 3.5.4. using the following controls:

1. Transfection with all constituents except the transfer plasmid

2. Transfection with all constituents except the linear baculovirus DNA
3. Transfection with media only.

5.4.3 UV microscopy

Sf9 cell sheets were analysed for recombinant GFP baculovirus by viewing infected cell sheets using a UV microscope equipped with a Fluorescein filter.

5.4.4 Light microscopy

Sf9 cell sheets were analysed for recombinant F2, baculovirus by viewing infected cell sheets using an inverted light microscope.

5.4.5 Plaque purification

GFP and F2 recombinant baculovirus were plaque purified as described in section 3.5.4

5.4.6 PCR analysis of plaque purified virus

DNA was extracted using the Qiagen blood sample protocol supplied with QiAmp DNA extraction kits (Qiangen, UK 51304). DNA was analysed using standard Taq PCRs (3.3.1)

5.4.7 Insect cell lysis and his tag purification

Sf9 cells were lysed and purified as follows:

Approximately 3.3 x 10^6 cells (1 x 25cm^2 cell culture flask) were re-suspended in 600µl of lysis cocktail (see below) followed by 5 cycles of freeze thawing. Unclarifed lysates were centrifuged at 1000 x g for 10minutes, separating insoluble material. Clarified lysates were filtered using 0.2µm syringe filters before his tag purification. Native his tag purification was used as described in

section 3.4.3. Insoluble material was re-suspended in 1ml of CBC buffer containing 8M urea.

Lysis cocktail

10µl protease inhibitor (Sigma, P 8849)

15µl triton x (Sigma, T8787) (10% in PBS)

7.5µl DNAse (Sigma, D5025)

Made up to 1ml in the following buffer:

Lysis buffer

0.535g $NaH_2PO_4 + 2H_2O$

3.07g Na_2HPO_4

8.77g NaCl

0.7g Imidazole (Sigma 10250)

Made up to 500ml with distilled water.

5.4.8 ELISAs

Sf9 insect cell lysates and his tag purified products were prepared and tested in his tag ELISAs as described in section 5.3.9

5.5 Results part 1: Developing clones

5.5.1 Amplification of APV, GFP and APV-GFP genes

Figures 43, 44 and 45 show N, P, F and GFP Pfu PCR products that had been amplified from the full length clone, N and P-PCI clones, F-p18smahis and GFP-p18smahis using newly designed oligos given in tables 2, 3 and 4.

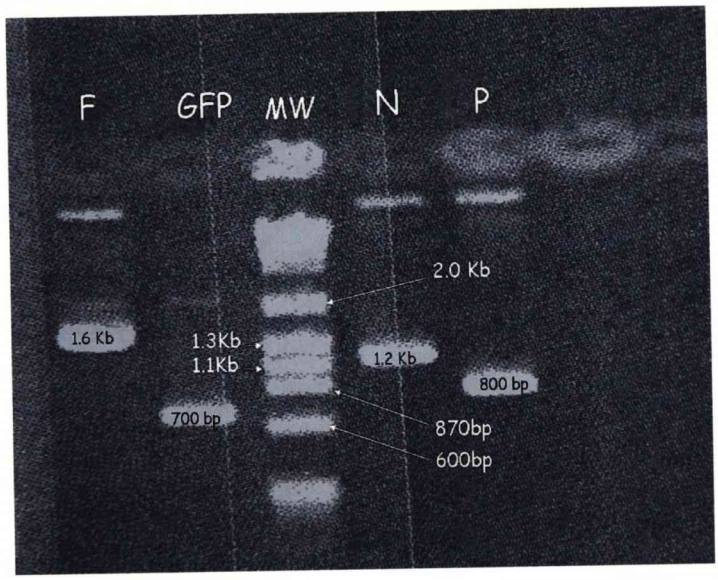


Figure 43. N, P and F, amplified from an APV full length clone and GFP from the GFP-p18smahis clone developed in Chapter 4. (agarose gel, stained with ethidium bromide)

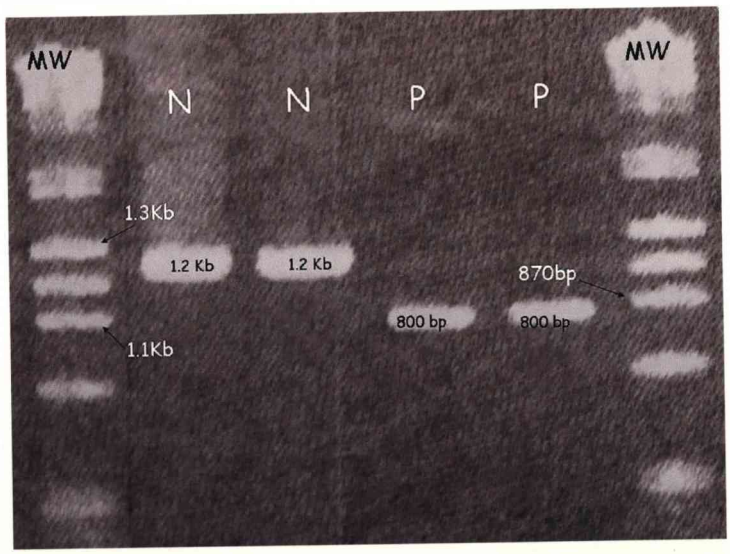


Figure 44. Duplicate N and P PCR products amplified from N and P PCi clones (agarose gel, stained with ethidium bromide)

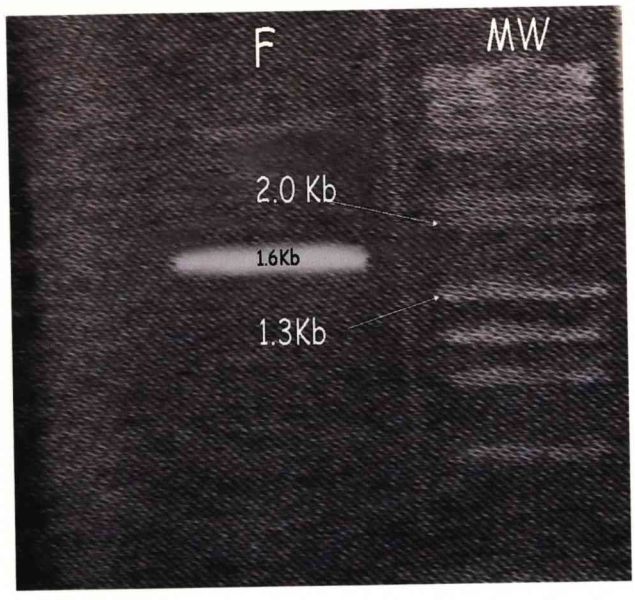


Figure 45. F amplified from F-p18smahis (agarose gel, stained with ethidium bromide)

Figure 46, shows N, P and F-GFP Pfu PCR products. These represent products illustrated at stage 4, Figure 41.

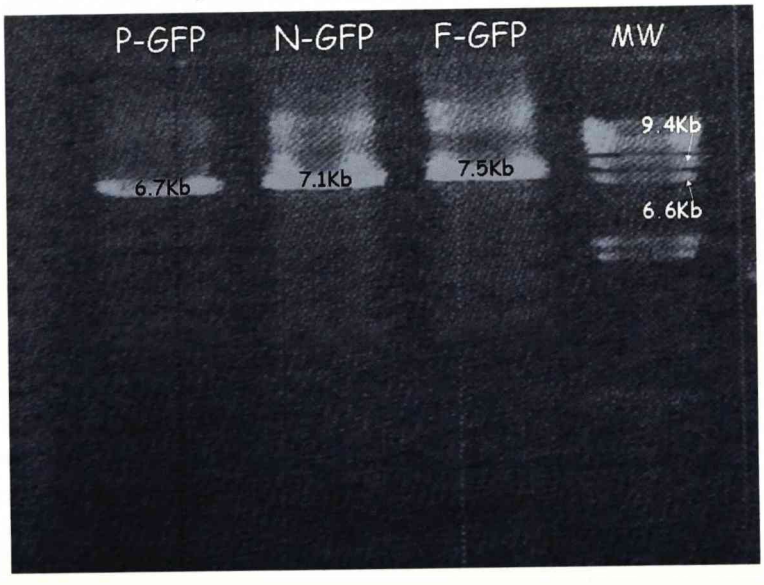


Figure 46. N, P and F-GFP PCR products (agarose gel, stained with ethidium bromide

Figure 47, shows N and F-GFP Pfu PCR products that were generated in a "Quick Change" SDM reaction. These constructs contained genes that would produce both N and COOH terminal his tagged recombinant proteins.

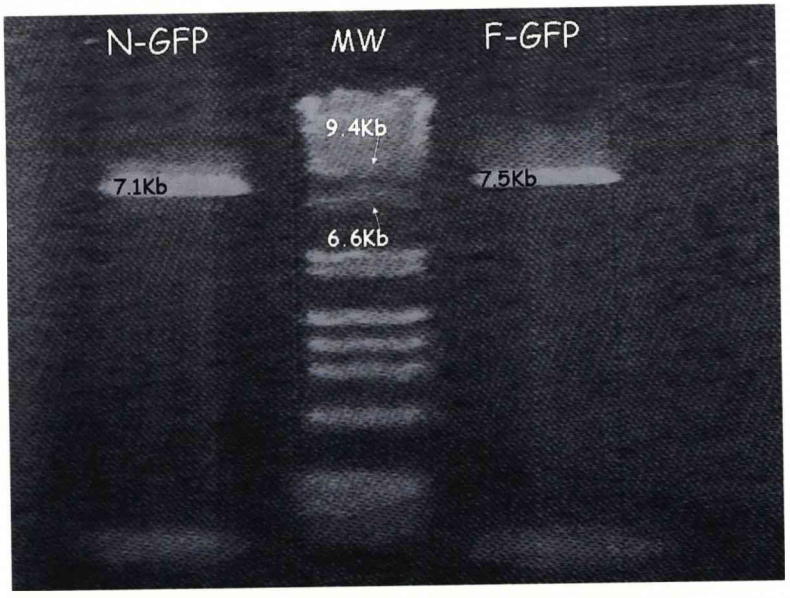


Figure 47. N, P and F-GFP, PCR products produced in an SDM reaction (agarose gel, stained with ethidium bromide)

5.5.2 Amplification of the APV F gene and the GFP gene for baculovirus expression

Figure 48 shows PCR products that represent both the F gene and the GFP gene. These had been amplified from F and GFP-p18smahis vectors using oligos listed in table 7. Each oligo introduce specific Mfe 1 RE recognition sites to both the 5' and 3' ends.

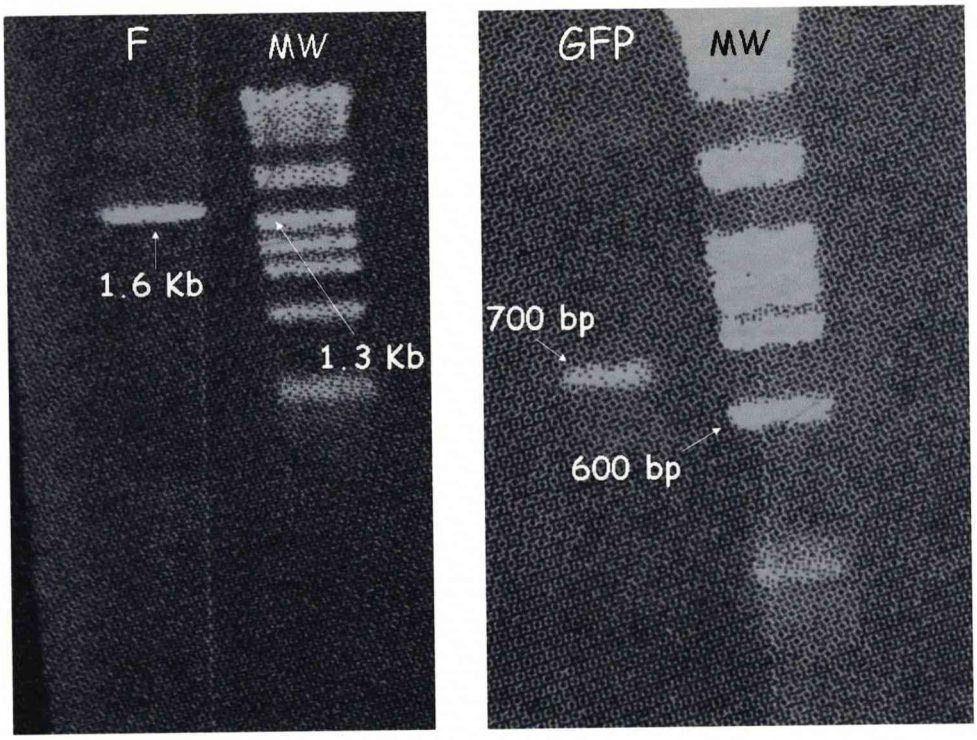


Figure 48. F and GFP PCR products amplified in a Pfu PCR using oligos that introduced specific Mfe 1 RE recognition sites to both the 5' and 3' ends (agarose gel, stained with ethidium bromide)

5.5.3 Identification of clones with the correct sequence

After ligating the products shown in Figures 43-48 with specific plasmid vectors (see 5.2.3) and subsequent transformation of *E.coli* cells, Taq PCR and restriction endonucleases screening methods (3.3.1 and 4.6.2) (3.3.6) identified several positive clones. Plasmid ligations with APV gene products amplified from p18smahis and PCI produced successful clones; however, no success was achieved using products amplified from the full length clone of Naylor et al. [72] . Extracted plasmids were then sent for sequencing and those with the correct nucleotide and predicted amino acid sequence were used for *E.coli* and insect cell expression. Several clones of each construct were identified with the exception of P-GFP in p-ET 30, and F1, and F1/tail in pBlueBacHis2. Figures 49 and 50 are examples of N and P p-ET 30 clones containing the correct sequence (analyse using Generunner 3.05).

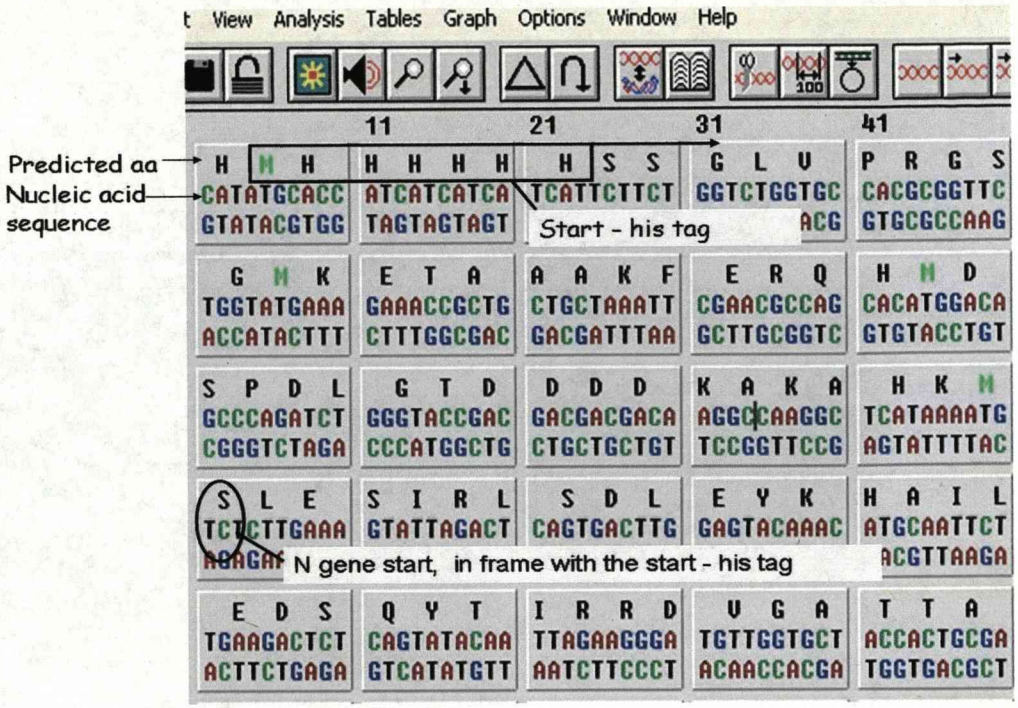


Figure 49. Nucleic and predicted amino acid sequence of the APV N gene in p-ET 30 with an N terminal his tag region

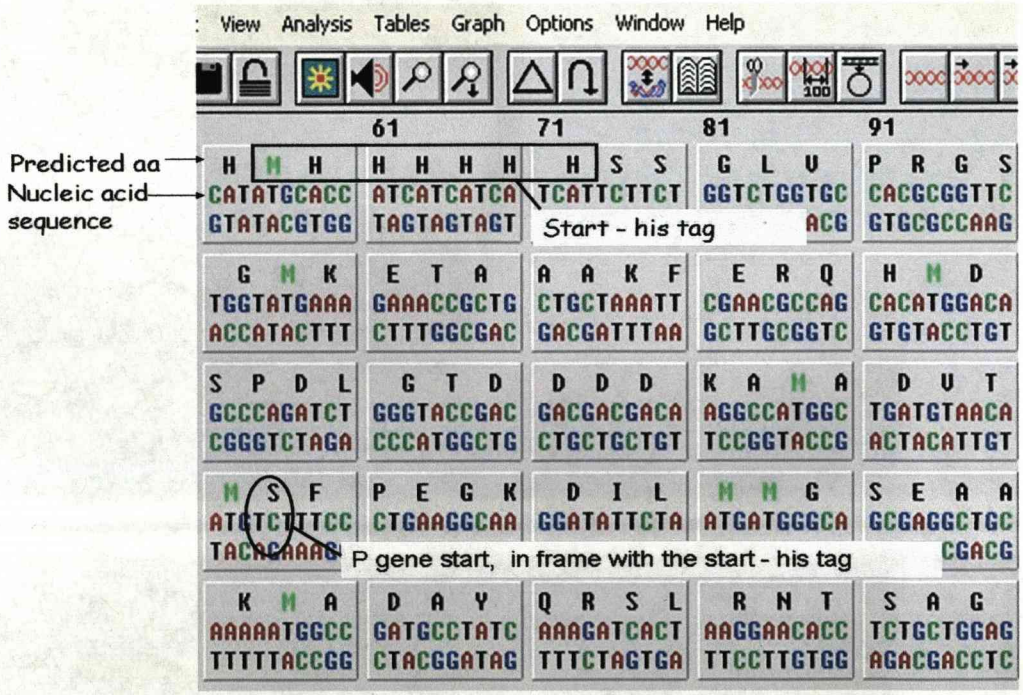


Figure 50. Nucleic and predicted amino acid sequence of the APV P gene in p-ET 30 with an N terminal his tag region

5.6 Results part 2: *E.coli* expression

~95% of transformed BL21 (DE3), BL21 (DE3) pLysS and BL21 (DE3) pLysE *E.coli* cells, yielded clones of each construct. These were identified using the Taq PCR method (4.6.2). However, colonies containing the GFP construct could be identified visually without the need for PCR analysis. Figure 51 shows fluorescent, BL21 (DE3) pLysE GFP colonies viewed over a UV transilluminator.

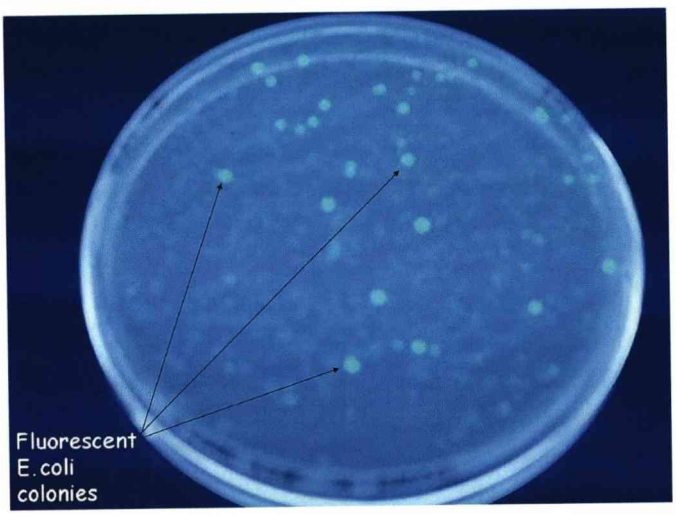


Figure 51. Un-induced, fluorescing BL21 (DE3) pLysE cells that contained the GFP-pET30 construct. Plate was viewed over a UV transilluminator.

5.6.1 GFP and N expression

The first clones N and GFP in BL21 (DE3) pLysE cells were induced with 1mM of IPTG for a total of 3 hours. A non-induced control and 3 samples, taken at 1 hour intervals from the GFP clones, were analysed using UV microscopy. Figure 52 demonstrates an increase in GFP expression over time.

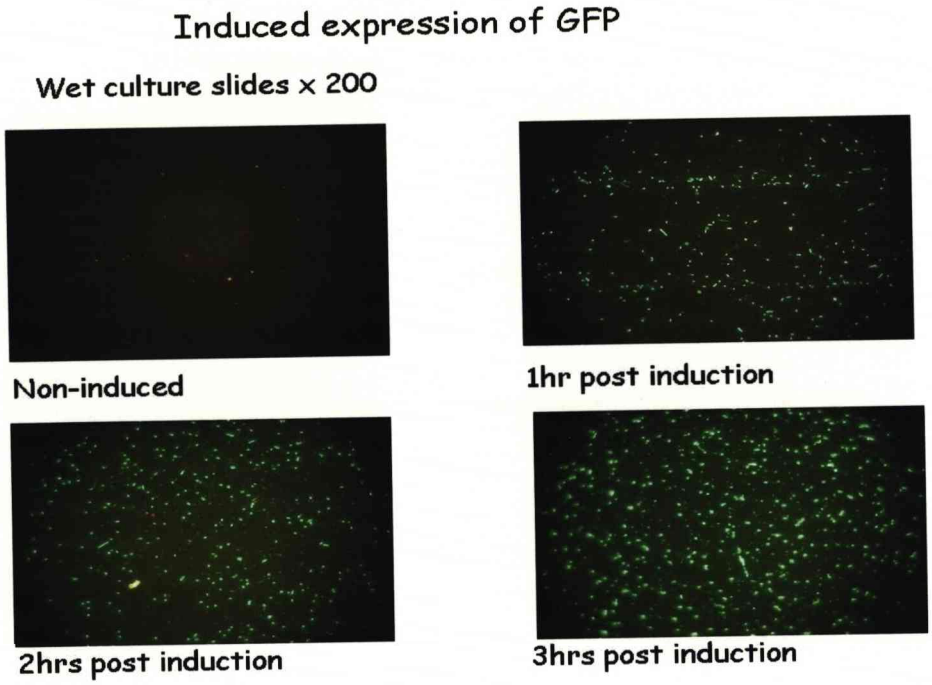


Figure 52. Un-induced and induced GFP cultures. An increase in GFP expression over a period of 3 hours, viewed using UV microscopy.

Samples taken at 3 hours post induction for both N and GFP were used in denaturing and native Ni-NTA purification. The products following purification were analysed on SDS-PAGE gels shown in Figures 53 and 54

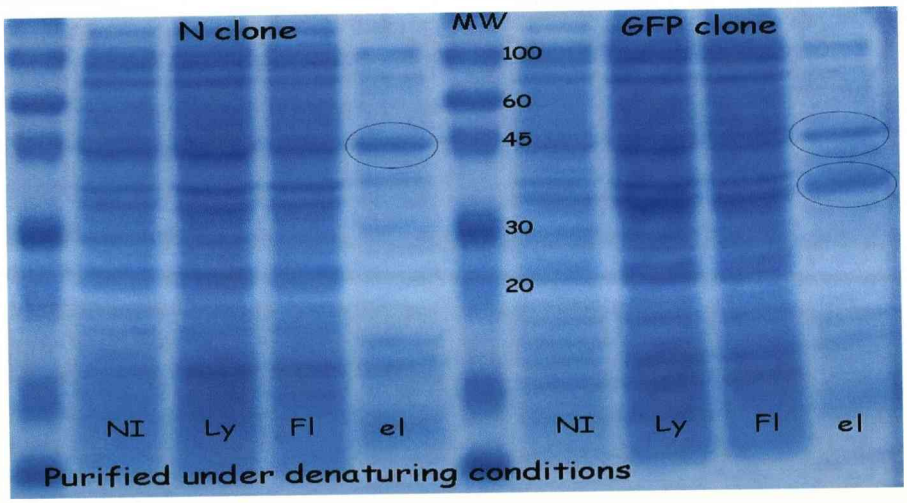


Figure 53. SDS-PAGE analysis of induced N and GFP clones, purified under denaturing conditions **NI** = non-induced control, **Ly** = cell lysate, **Fl** = flow through, **el** = purified sample.

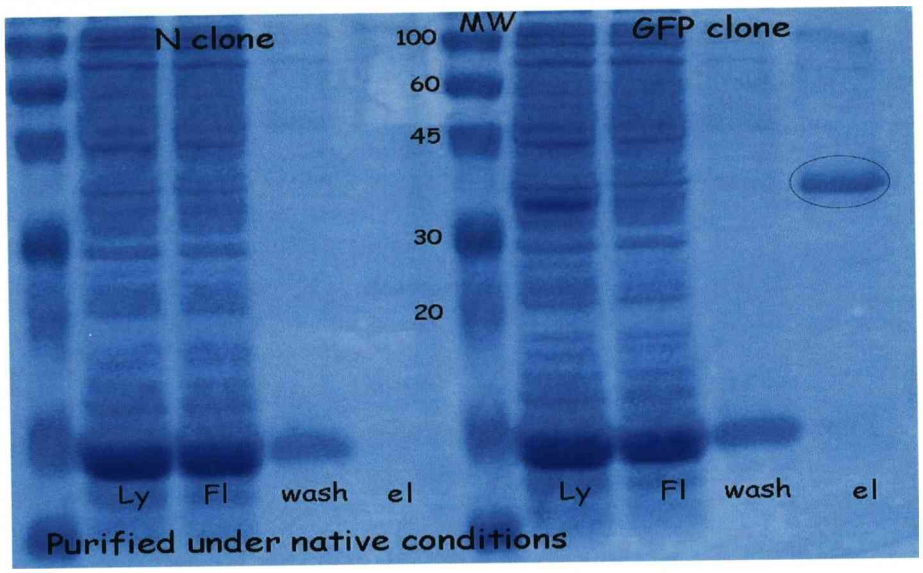


Figure 54. SDS-PAGE analysis of induced N and GFP clones, purified under native conditions **Ly** = cell lysate, **Fl** = flow through, wash fraction, **el** = purified sample.

Under denaturing conditions purified N produced a band of ~44KDa (highlighted Figure 53) which was close to published sizes 38-43KDa [16]. However, a protein of the same size was observed in the purified GFP sample (Figure 53). As this was too large to represent GFP and appeared in both samples, it most likely represented an *E.coli* contaminant. Absence of similar bands in a western blot of these samples, using polyclonal APV positive turkey serum confirmed this (data not shown). No reactions were observed with any test samples.

Purified GFP produced two bands under denaturing conditions: the fore mentioned 44KDa band and a band of ~31KDa (Figure 53). This 31KDa band correlated with the published GFP size of Ormo *et al* [220]. Under native conditions the 44KDa band did not appear, only the expected 31KDa GFP band was observed (Figure 53). In addition, GFP fluoresced at each stage in the purification procedure. No purified N protein was observed under native conditions.

These gels showed GFP successfully his tag purified under standard native and denaturing conditions and native purification producing less contaminants. Purification of N protein from BL21 (DE3) pLysE under standard native and denaturing conditions failed. In addition, a general observation when using standard denaturing conditions was the increase of contaminating protein in purified samples.

5.6.2 Expression and purification of N, P and F from BL21 (DE3) cells

As previous attempts to purify N protein using BL21 (DE3) pLysE cells had failed the next set of clones analysed were BL21 (DE3) cells. Expression levels are higher in these cells and therefore may have been beneficial to purification. Each construct had been induced using 0.5mM IPTG for a period of 3 hours. All had been purified under both standard native and denaturing conditions. In addition to the standard purification was a modified native purification protocol; 8M urea was added to native lysates and native wash and elution buffers. This would determine if native soluble protein was being expressed but not captured due to concealment of his tag regions. Essentially, a high concentration of urea would linearise soluble native protein and expose any concealed his tag regions that may have been hindering Ni_2 + capture.

Before purified products were analysed, native and denatured cell lysates of induced N, P and F clones (three separate clones for each) were examined by SDS PAGE (Figure 55). A comparison of these lysates revealed a significant difference in the P clone. This clone produced a highly concentrated, 37KDa band in both native and denatured samples (highlighted in Figure 55) which fell

between the published sizes of 35-40KDa for APV P protein [16]. No significant differences could be observed for N, and F lysates.

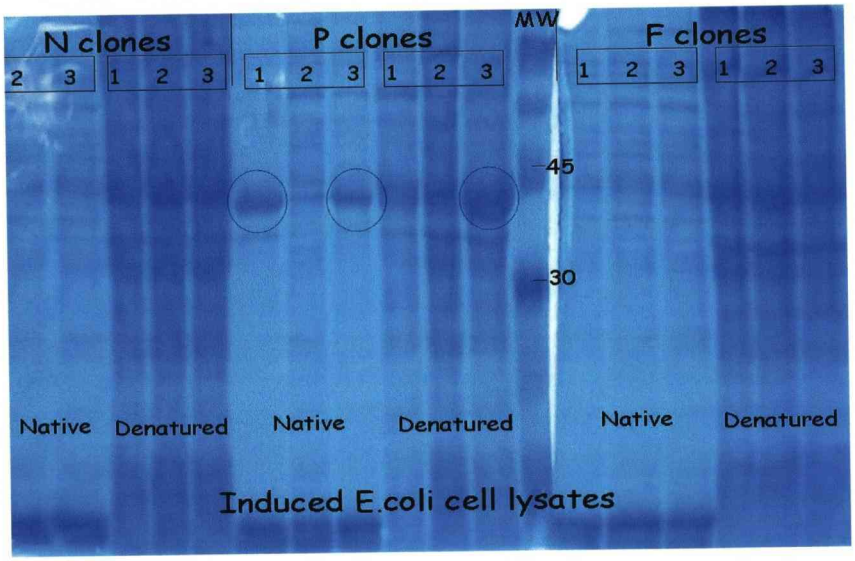


Figure 55. SDS-PAGE analysis of native and denatured cell lysates of induced N, P and F clones.

Figure 56 shows products that were purified from N, P and F lysates shown above using standard native and denaturing purification techniques (3.4.3).

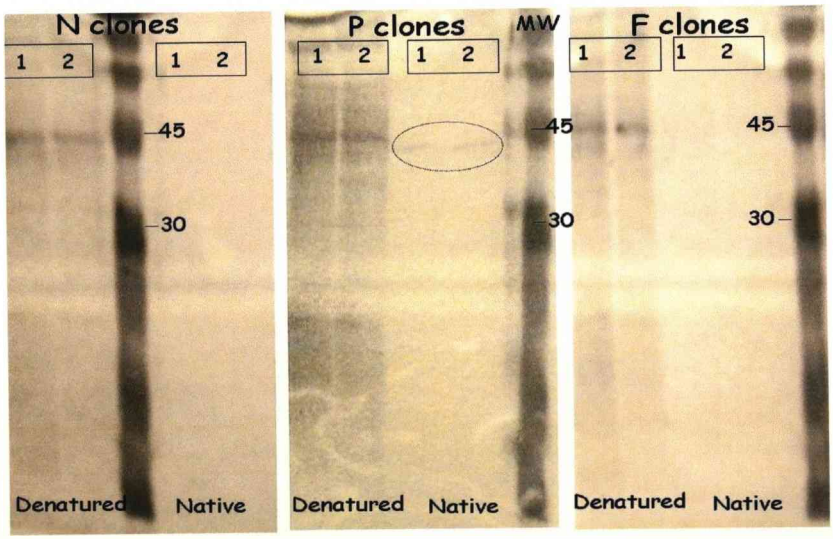


Figure 56. SDS-PAGE analysis of N, P and F purified products following native and denaturing his tag purification. Numbers 1 and 2 represent individual clones

A 37KDa protein (highlighted figure 56) was observed in both purified native P samples. This protein was absent in N and F purified samples, as it was with cell lysate analysis (Figure 55) and therefore probably represented recombinant APV P protein. No recombinant N or F proteins were observed in native samples and all denatured samples were again, highly contaminated. Contamination could have resulted from proteins associating with his tag regions and co-purifying as a result. To address this, a modified purification protocol was applied. In this experiment a denatured lysate (number 1, N clone Figure 55) had been purified in the presence of constituents that would prevent such associations; namely, 2me, ethanol and glycerol (modification 1, 2 and 3. section 5.3.5.). Comparisons were made against a standard purification using SDS PAGE. None of these changes improved the clarity of the purified sample. It was considered that some *E.coli* proteins may have had internal regions of consecutive histidine amino acids which were only exposed under denaturing conditions. This would allow them to bind specifically with Ni_2+ charged columns, creating a binding site competition with recombinant proteins. As a result a range of unwanted proteins would be purified.

A modified native purification (modification 4. section 5.3.5.) of N, P and F resulted in the SDS PAGE gel shown in Figure 57. Both N and P produced bands of expected sizes in purified samples (~43KDa and 37KDa respectively) (highlighted Figure 57) under these conditions and appeared to have a low level of contamination. As postulated, native recombinant N and P protein was being expressed, but capture was hindered due to concealment of his tag regions. No purified F protein was observed. Samples suspected of containing recombinant

proteins were tested in APV and his tag ELISAs (5.2.4).

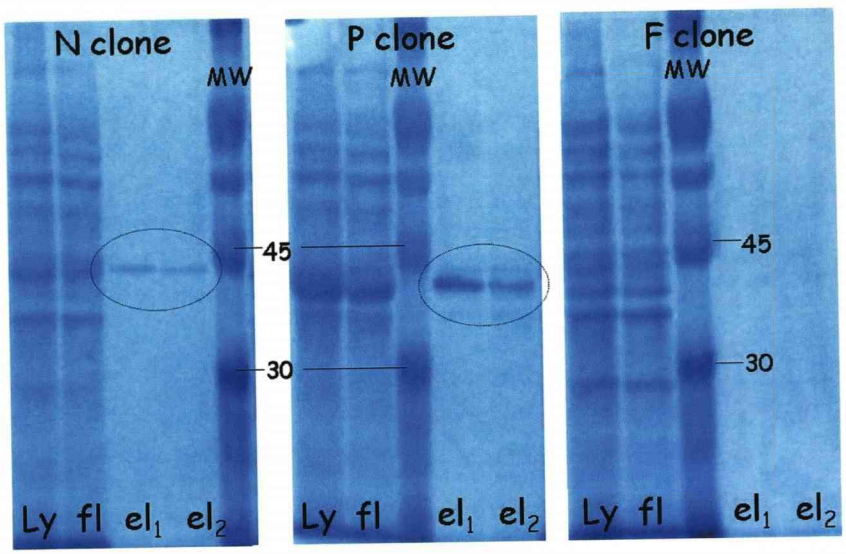


Figure 57. SDS-PAGE analysis of N, P and F; purified under modified native conditions: **Ly** = cell lysate, **Fl** = flow through, **el_1** = first fraction of the purified sample **el_2** = second fraction of the purified sample

5.6.3 Expression and purification of APV-GFP constructs

N-GFP and F-GFP clones (N-terminal his tagged) in BL21 (DE3) cells that had been induced with 0.5mM of IPTG for a total of 4hours were first analysed using UV microscopy. Figures 58 and 59 show wet culture slides of N-GFP and F-GFP. An increase in fluorescent intensity was observed over the four hour induction period for both. However, cells appeared to be losing structural integrity and fluorescence was diffused compared with the same cells expressing GFP alone (Figure 60). *E.coli* appeared to have a low tolerance of these proteins.

N-GFP and F-GFP clones (N, and COOH-terminal his tagged) produced the same images.

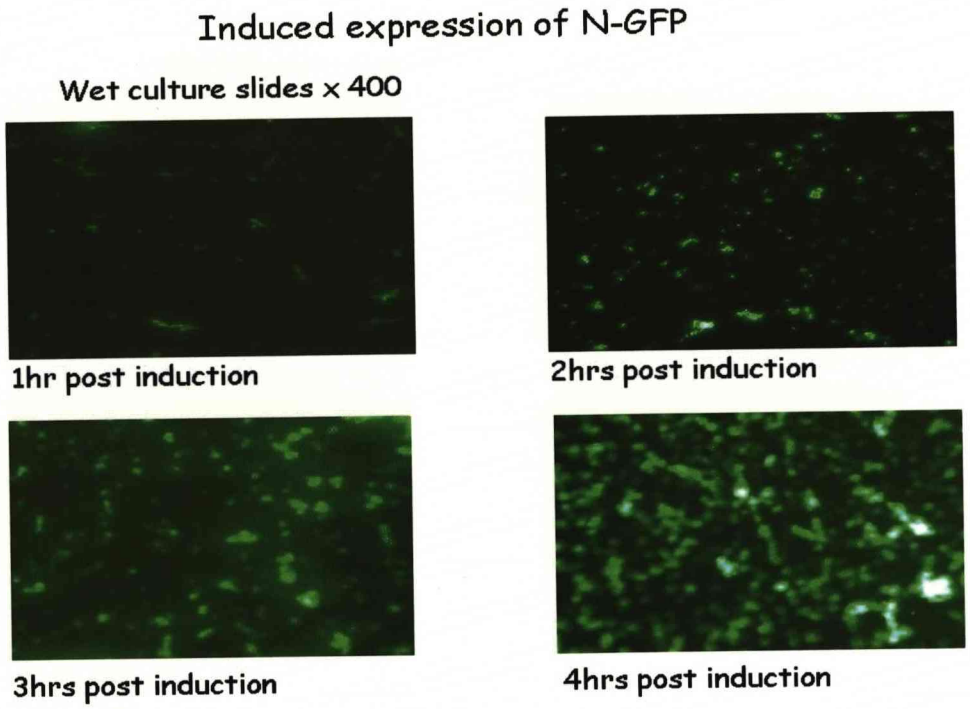


Figure 58. Induced N-GFP cultures. An increase in expression over a period of 4 hours, viewed using UV microscopy.

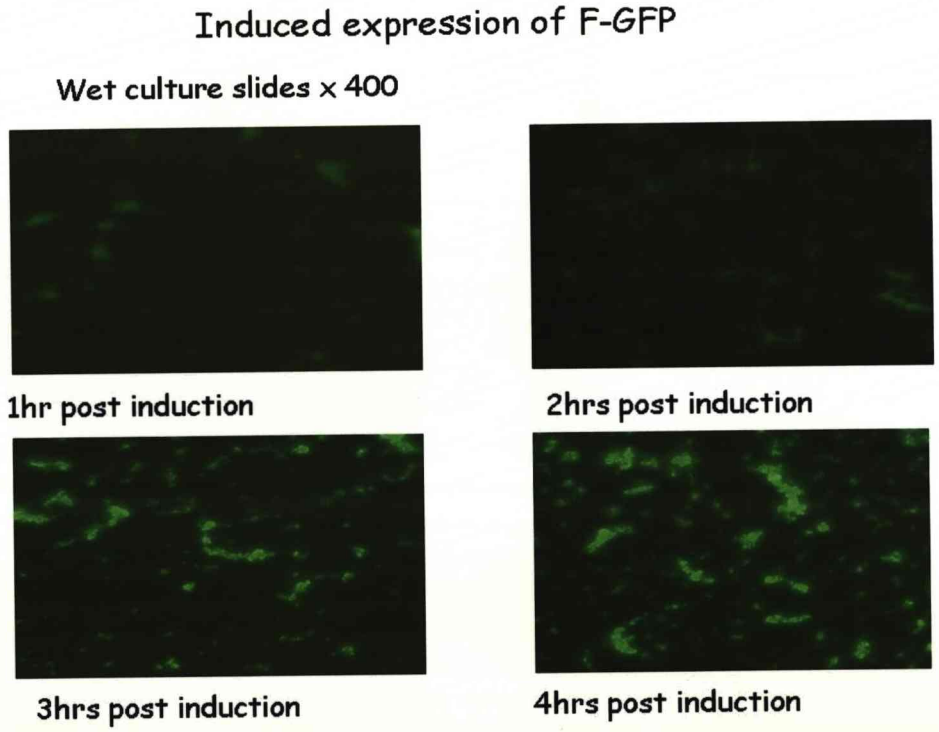


Figure 59. Induced F-GFP cultures. An increase in expression over a period of 4 hours, viewed using UV microscopy.

Comparisons between Induced *E.coli* cells either containing N-GFP, F-GFP or GFP alone. x400

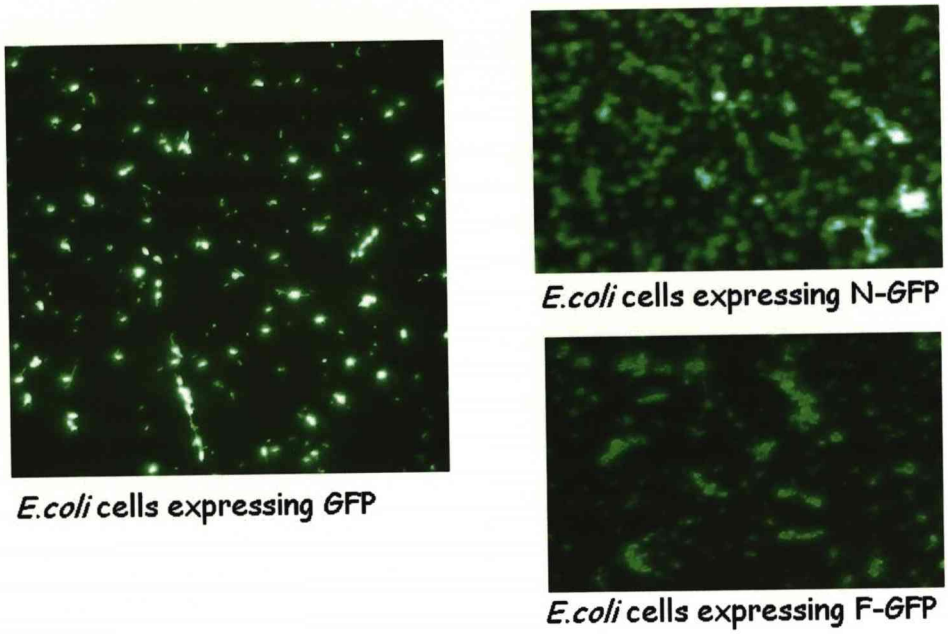


Figure 60. Induced N-GFP, F-GFP and GFP cultures viewed using UV microscopy. Cells expressing N or F-GFP appeared to be losing their structural integrity and fluorescence was diffused compared with those expressing GFP alone.

Each APV-GFP fluorescent culture was used for standard native purification and analysed using SDS PAGE. No proteins of correct sizes were observed in purified samples. However, his tag western blot analysis of N-GFP and F-GFP (his tagged at both the N and COOH terminal) cell lysates, produced N-GFP specific bands that correlated to the approximate size of N plus GFP = 76KDa (highlighted Figure 61). F-GFP lysates produced 32KDa bands (highlighted Figure 61) however; the approximated size for F-GFP was 90KDa. The 32KDa band may have represented the GFP section following proteolytic cleavage from F. This section would retain the COOH his tag for detection. Sizes were calculated by plotting the Rf values of highlighted samples against known standards as described in section 4.7.6.

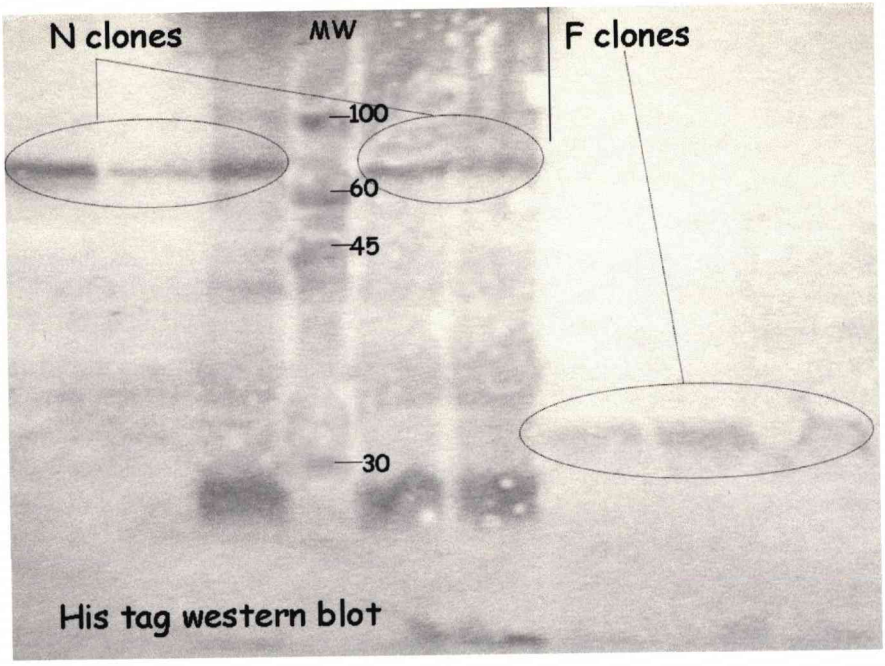


Figure 61. his tag western blot of native N-GFP and F-GFP cell lysates
N-GFP highlighted bands = 76KDa and F-GFP = 32KDa

his tag Western blot demonstrated specific his tagged proteins in cell lysates of dual tagged N-GFP and F-GFP however, native his tag purification was unsuccessful. Concealment of his tag regions was again suspected.

5.6.4 APV and his tag ELISAs of *E.coli* expressed products

Denatured N protein and native N protein purified in the presence of 8M urea were tested in conjunction with native P protein and native P protein purified in the presence of 8M urea in an APV ELISA. Results of this ELISA are shown in Figure 62.

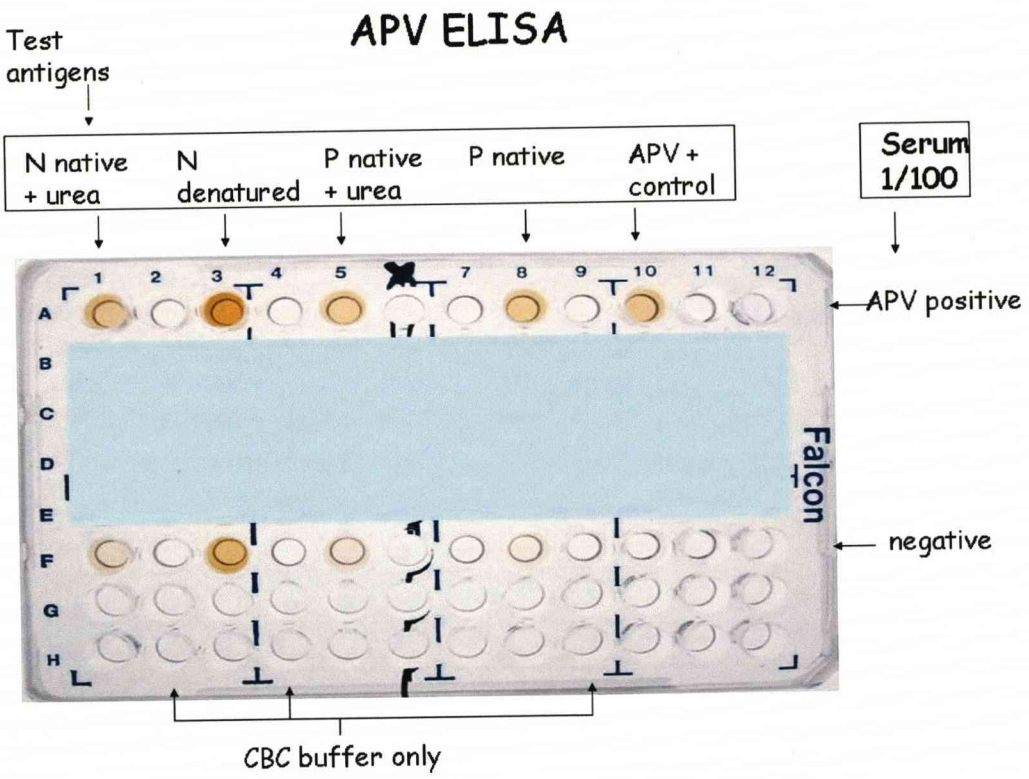


Figure 62. APV ELISA of *E.coli* expressed N and P

Each antigen reacted with APV positive serum (row A lanes 1, 3, 5, 8 and 10 Figure 62). However; N and P reactions with negative control serum were also apparent (row F lanes 1, 3, 5 and 8 Figure 62). Reactions with negative serum were stronger with N antigens and N products purified under denaturing conditions in particular. Samples purified under denaturing conditions contained consistent high levels of contaminating proteins when observed by SDS PAGE analysis so were also anticipated to effect ELISAs. It was considered that *E.coli* specific antibodies in test serum, reacting with *E.coli* contaminants in test samples, were responsible. Compared with APV standard control antigen, all test antigens had increased background.

To confirm the presence of recombinant his tag protein, the above antigens were tested in conjunction with native F (purified in the presence of 8M urea) in a his tag ELISA. Native GFP (Figure 54) was used as a positive control and *E.coli* lacking recombinant his tag protein was used as a negative control. Results of this ELISA are shown in Figure 63.

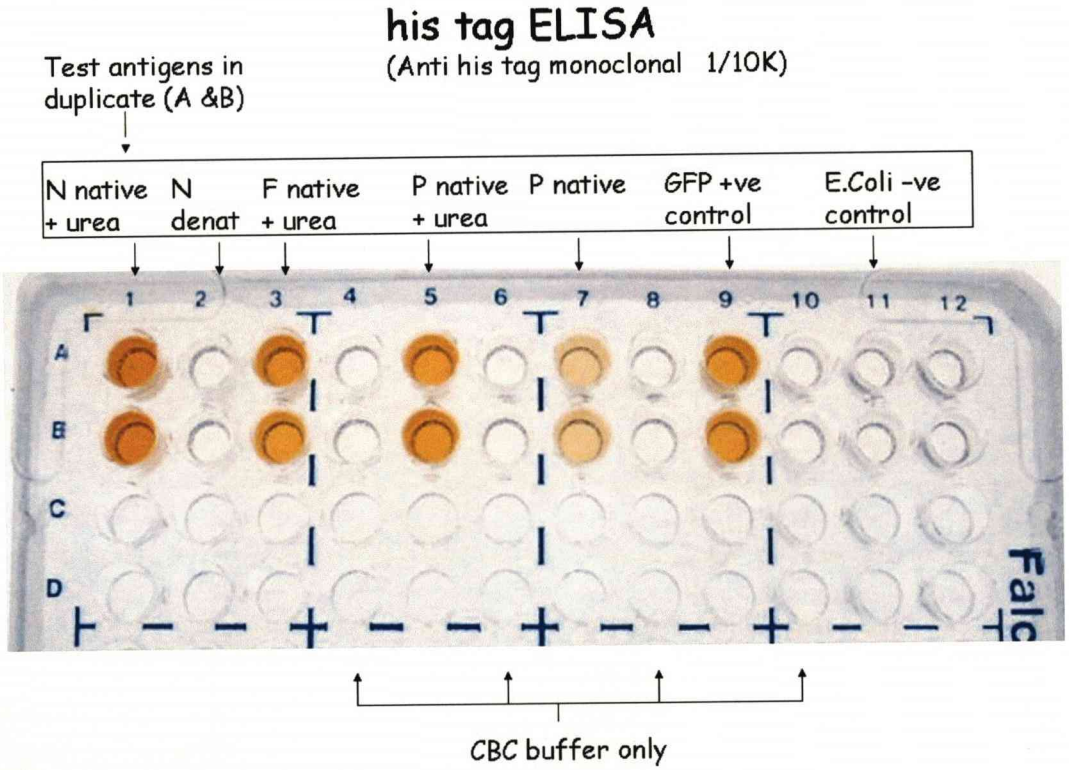


Figure 63. his tag ELISA of *E.coli* expressed N, P, F and GFP

Both controls functioned as anticipated and clear positives were observed with each antigen purified in the presence of 8M urea (lane 1, 3 and 5 Figure 62) and with native P protein (lane 7 Figure 5). N protein purified under denaturing conditions gave a negative result. In agreement with SDS PAGE analysis it appeared in these assays that native N, P and F proteins purified in the presence

of 8M urea yielded an increased amount of purified protein. However as linear proteins they were not suitable APV antigen candidates as previously discussed.

5.7 Results part 3: Baculovirus expression

5.7.1 Identification of recombinant baculovirus

Between days 4 and 7 post transfection, Sf9 insect cells were analysed for recombinant GFP and F2 baculovirus. GFP cell sheets were viewed using UV microscopy and F2 cell sheets were viewed using light microscopy. Under light microscopy cells containing recombinant baculovirus are free of occlusion bodies which appear as refractive crystals in cell nuclei. In cells infected with wild type virus there is a general increase in cell diameter. The results of these analyses are shown in Figures 64 and 65.

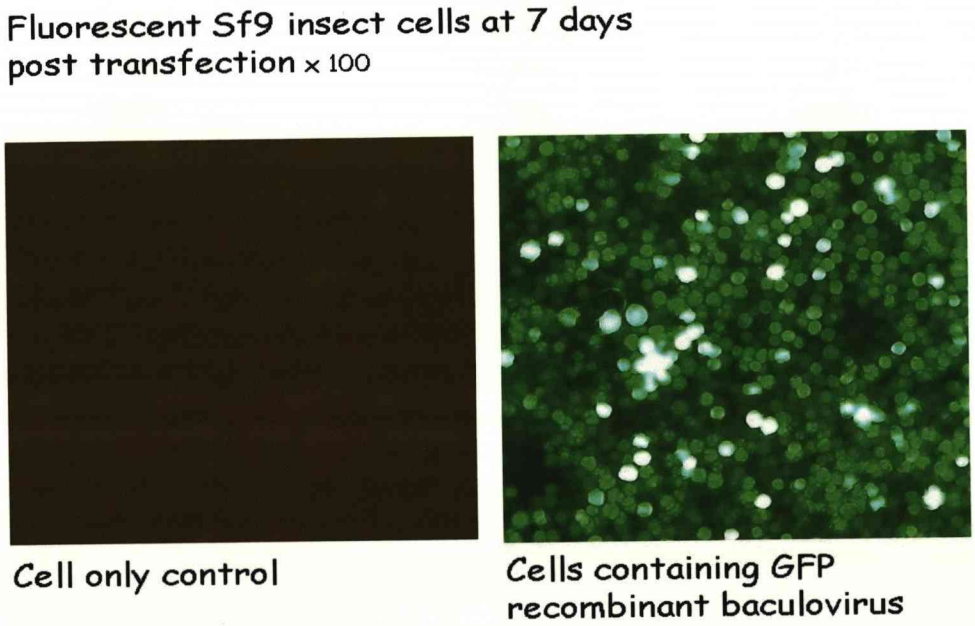


Figure 64. Transfected Sf9 insect cells containing GFP recombinant baculovirus. Intense fluorescence observed at 7 days post transfection compared with cell only control.

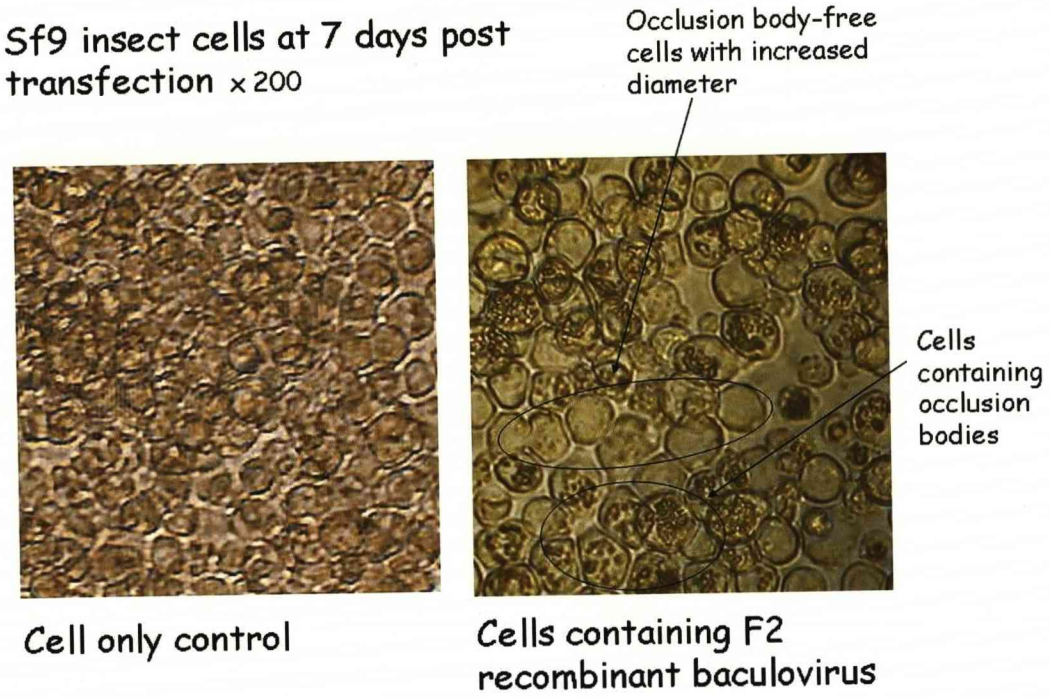


Figure 65. Transfected Sf9 insect cells containing F2 recombinant baculovirus. Cells with and without occlusion bodies were observed at 7 days post transfection, indicating presence of both recombinant and wild type virus. Transfected cells had increased diameter compared with the cell only control and were beginning to detach from the adherent monolayer.

Cells containing occlusion bodies (wild type virus) had been expected as transfected material was unlikely to produce 100% recombinant virus. GFP cell sheets also reviled areas of occlusion bodied cells when viewed using light microscopy. Importantly these areas were not fluorescent under UV light.

Recombinant GFP and F2 were analysed in the same way following plaque purification (Figure 66).

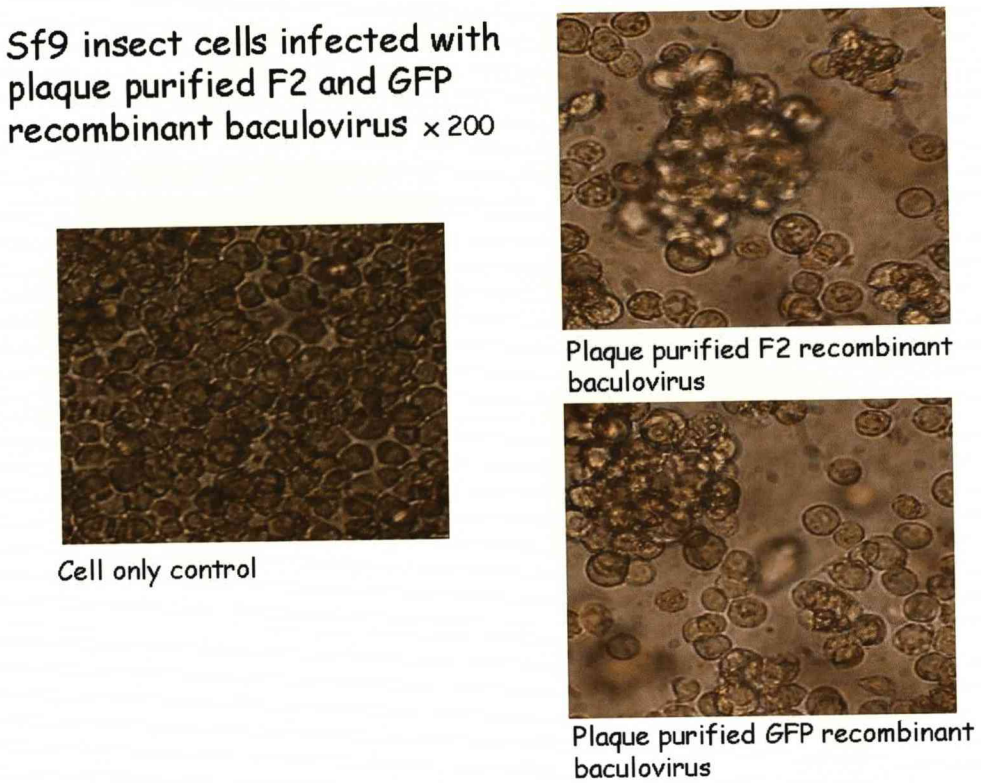


Figure 66. Sf9 insect cells infected with plaque purified GFP and F2 recombinant baculovirus. No occlusion bodied cells were observed and both infected cell sheets contained detached cells with increased diameter compared with the cell only control.

Plaque purification appeared to be successful as no cells were observed with occlusion bodies in either GFP or F2 infected Sf9 cell sheets and signs of infection were retained. This was confirmed by PCR analysis for F2 and by UV microscopy for GFP. Only DNA extracted from plaque purified F2 baculovirus infected cells (absent of occlusions) produced the expected 137bp band (Figure 67) when compared to extracts of cells containing occlusion bodies (wild type virus) and from uninfected cells. Plaque purified GFP produced fluorescent cells identical to those shown in Figure 64 and therefore did not require PCR analysis.

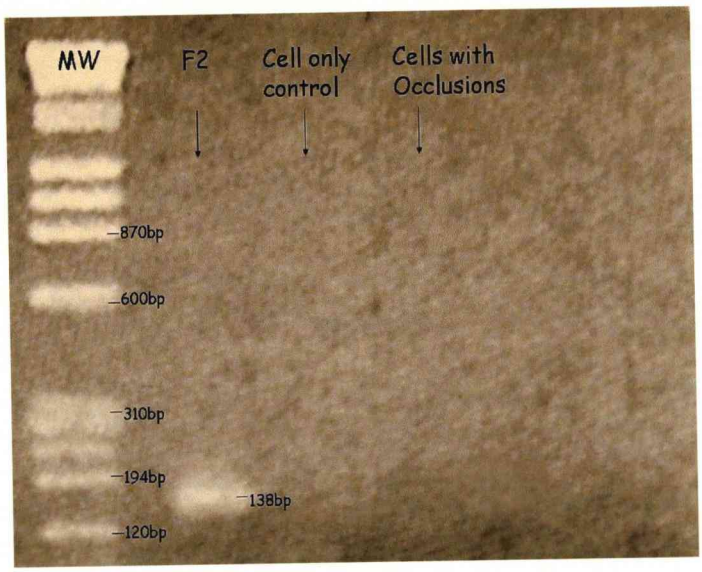


Figure 67. PCR analysis of DNA extracted from Sf9 cells infected with plaque purified F2 recombinant baculovirus, cells with occlusion bodies and uninfected cells.

5.7.2 his tag purification

His tag ELISAs were used to analyse F2 native purified samples and F2 and GFP native lysates. These are shown in Figures 68 and 69

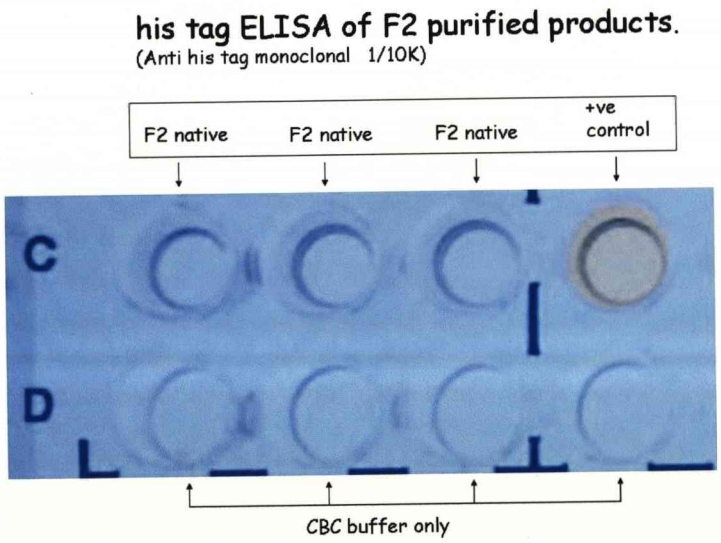


Figure 68. his tag ELISA of native F2 purified products that were prepared from F2 infected Sf9 insect cell lysates shown in figure 66.

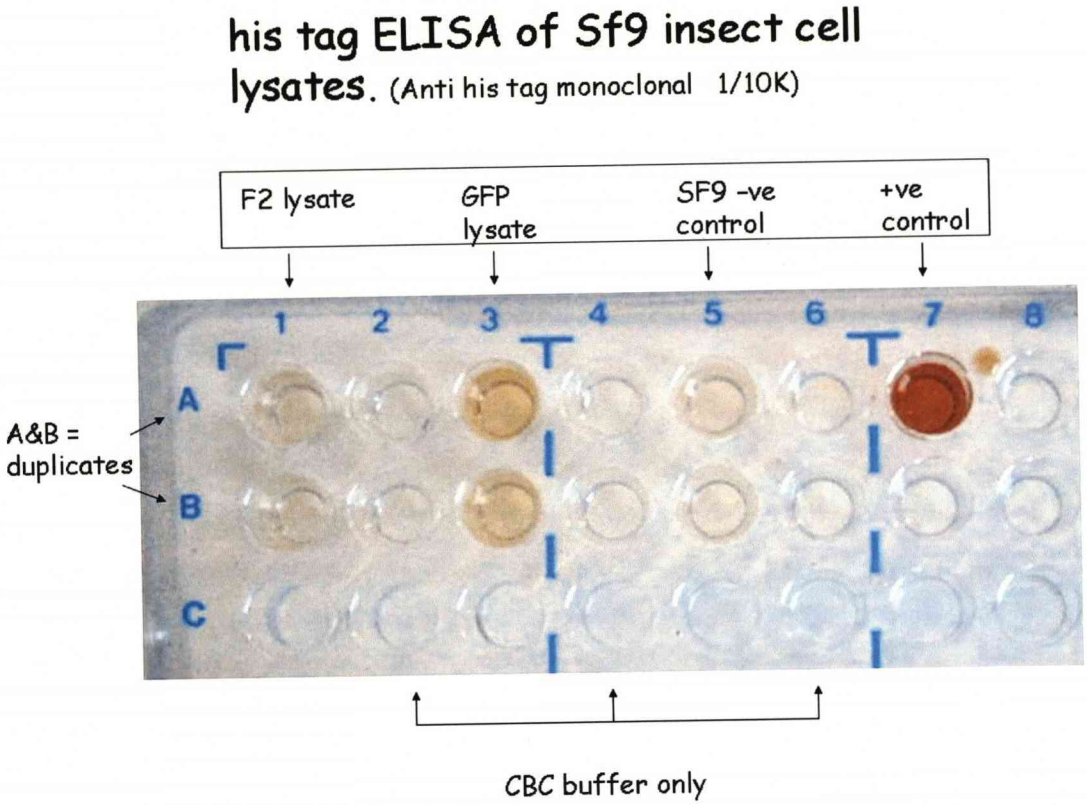


Figure 69. his tag ELISA of native F2 and GFP lysates prepared from infected Sf9 insect cells.

Only the positive controls and the GFP lysates reacted in these ELISAs (C +ve control Figure 68 and lane 3 and 7 Figure 69) and no his tag protein was detected in the F2 lysate or purified samples. It was considered that the hydrophobic signal peptide at the start of F2 may have resulted in its association with other hydrophobic proteins in the expression cells, thus making it insoluble under native conditions. Immediate N-terminal signal peptide regions (as in the full length F protein) are usually removed by signal peptidases however, as the signal peptide of F2 recombinant protein was preceded by a his tag region, it would remain intact.

Insoluble pellets that remained following native lysis were re-suspended using the method given in section 5.4.7. This material was then analysed for the presence of recombinant F2 protein in a his tag ELISA (Figure 70). Insoluble material (re-suspended in the same way) from non infected cells was used as a negative control.

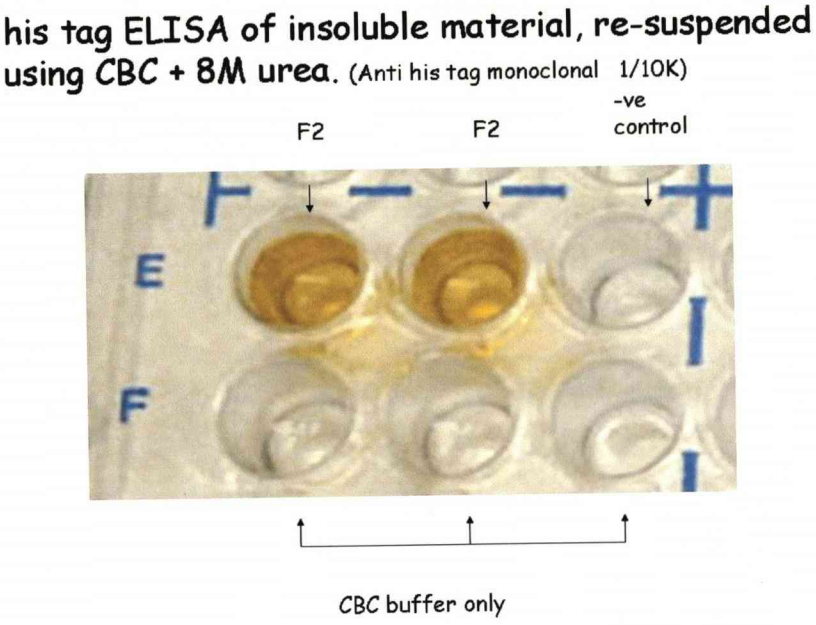


Figure 69. his tag ELISA of F2 his tag recombinant protein prepared from the insoluble material of lysed, F2 baculovirus infected Sf9 insect cells.

Both F2 samples (extracted from insoluble cellular material) were positive by his tag ELISA and the negative control functioned accordingly. As postulated baculovirus expressed F2 recombinant protein was insoluble under native conditions; an implication that would render any purified protein ineffective as antigen due to the denaturing processes needed to extract them.

5.8 Discussion

Commercial expression vector p-ET30 readily accepted APV and GFP genes into its sequence and produced stable clones in DH5α. However, successful clones were only identified from ligations involving gene products amplified from existing clones that had different antibiotic resistance to that of p-ET30 (p18smahis and PCi). This suggested that many cells following transfection actually contained unwanted or original template plasmids. pBlueBacHis2 baculovirus transfer vector appeared to be less efficient as only one of three F sections and GFP were successfully cloned. This observation demonstrates the empirical nature of identifying plasmids that readily accept a chosen gene. The strategy illustrated in Figure 41 produced N and F-GFP fusion constructs efficiently with all of the clones screened containing the correct nucleotide sequences. In contrast, all P-GFP sequences contained at least one coding base substitution. These were distributed randomly throughout the gene.

Significant increase in expression of recombinant proteins were observed using p-ET30 compared to the developed p18smahis vector (Chapter 4) and this was best demonstrated by GFP. Fluorescence was observed pre-induction in a selection of clones (Figure 51) which was attributed to "leakage". BL21 (DE3) pLysE cells contain plasmids that express T7 lysosyme which digest T7 polymerases reducing basal level expression; however, some cells lose these plasmids during growth and therefore the property is lost and basal level expression can occur. Following IPTG induction of GFP an increase in fluorescent intensity was observed over a period of 3 hours and cells appeared structurally sound. In contrast, although an increase in fluorescent intensity of induced N and F-GFP was also seen, fluorescence was somewhat diffused.

Moreover, cells expressing N or F-GFP appeared to be losing structural integrity (Figure 60) which could suggest problems with APV protein toxicity in *E.coli*. Native GFP protein was successfully his tag purified and had low levels of contaminating proteins. However when purified under denaturing conditions contaminating proteins were observed (a later finding with all denatured samples). It was considered that some *E.coli* proteins may have had internal regions of consecutive histidine amino acids which were only exposed under denaturing conditions. This could have lead to specific *E.coli* protein purification using his tag capture. Purification of N and F-GFP was less successful. No purified N-GFP and F-GFP were identified; however a band that correlated with the calculated size of N-GFP was detected in native N-GFP lysates using a his tag western blot. As no protein was purified from this fluorescent lysate it was highly likely that concealment of its his tag regions was responsible. APV-GFP fusion proteins were useful for visual conformation of expression and may have provided an indication of APV protein toxicity problems in *E.coli*; however, purification was not improved whether tagged at the N terminal or both the N and COOH terminal.

Expression of N, P, and F was more difficult to assess visually by SDS PAGE compared to simple UV analysis of APV-GFP however, purification was more successful with these proteins. N, P and F proteins were purified using modified native conditions and a minimal amount of P protein was purified using standard native conditions. Although modified native purification produced linear protein which rendered them inappropriate as APV ELISA antigen candidates (as observed in Figure 62), it provided conformation that recombinant proteins were being expressed and that his tag purification could be achieved if his tag regions were exposed. His tag ELISAs used to detect these proteins were extremely

quick and efficient and would be used as a primary screening method for future detection.

Recombinant baculoviruses were developed and demonstrated a good level of expression as shown by GFP. However, recombinant F2 protein could not be purified under native conditions and was only demonstrated when insoluble material was re-suspended using a strong denaturant. Its insoluble properties were considered to be a result of the continued presence of a hydrophobic signal peptide region. This was not cleaved by signal peptidases due to the his tag making it inaccessible. PCR manipulations could be employed to remove this sequence generating new transfer vectors for new recombinant baculovirus production, however the generation of recombinant baculovirus is extremely time consuming compared with the generation of new *E.coli* clones therefore was not under taken.

A marked improvement in expression levels was generally observed using newly developed *E.coli* clones. However, APV-GFP clones produced visible changes in *E.Coli* morphology so suggesting problems with APV protein toxicity. In addition, purification procedures demonstrated concealment of his tag regions in native proteins. It was considered that expressing small regions of individual proteins might prove less toxic to *E.coli* and reduce concealment problems whilst retaining antigenic epitopes. Moreover, epitopes that were subtype specific could be identified.

Chapter 6

Expression and purification of sections of subtype-A APV fusion protein: Their use in ELISAs and potential importance in virus neutralization

Contents **Page**

Chapter 6

Expression and purification of sections of subtype-A APV fusion protein: Their use in ELISAs and potential importance in virus neutralization

6.1 Introduction

An indication of APV protein toxicity to *E.coli* (5.2.3, Chapter 5) and concealment of his tag regions in native protein following expression, led to the following hypothesis: If different regions of an individual protein were expressed separately: (i) problematic hydrophobic areas could be removed and (ii) reduced protein sizes could minimize his tag concealment issues. Moreover, this could allow identification of subtype-specific epitopes or epitopes common to all subtypes recognized by antibodies reacting in ELISAs and possibly those involved in virus neutralization as reported for RSV fusion protein by Lounsbach et al and Werle et al [188, 221].

The subtype A APV fusion protein was divided into 6 sections using the published F sequence of Naylor *et al* 1998 [48] as a design template. This is shown in Figure 70 along with one subtype B sequence and one subtype C sequence. Two sections were designed in the F2 region of the full length (F0) protein, three in the F1 region and one incorporating the entire cytoplasmic tail. The hydrophobic regions highlighted by Naylor *et al* 1998 [48] were excluded. Each section was amplified using oligos that maintained the same reading frame as shown in Figure 70 and incorporated an N-terminal his tag when cloned into *E.coli* expression vector p-ET30 (Figure 38, Chapter 5). Expressed protein was his tag purified.

This chapter describes the use of these six regions of the F protein as ELISA antigens and also investigates whether they could be targets for neutralizing antibodies in the sera of infected birds.

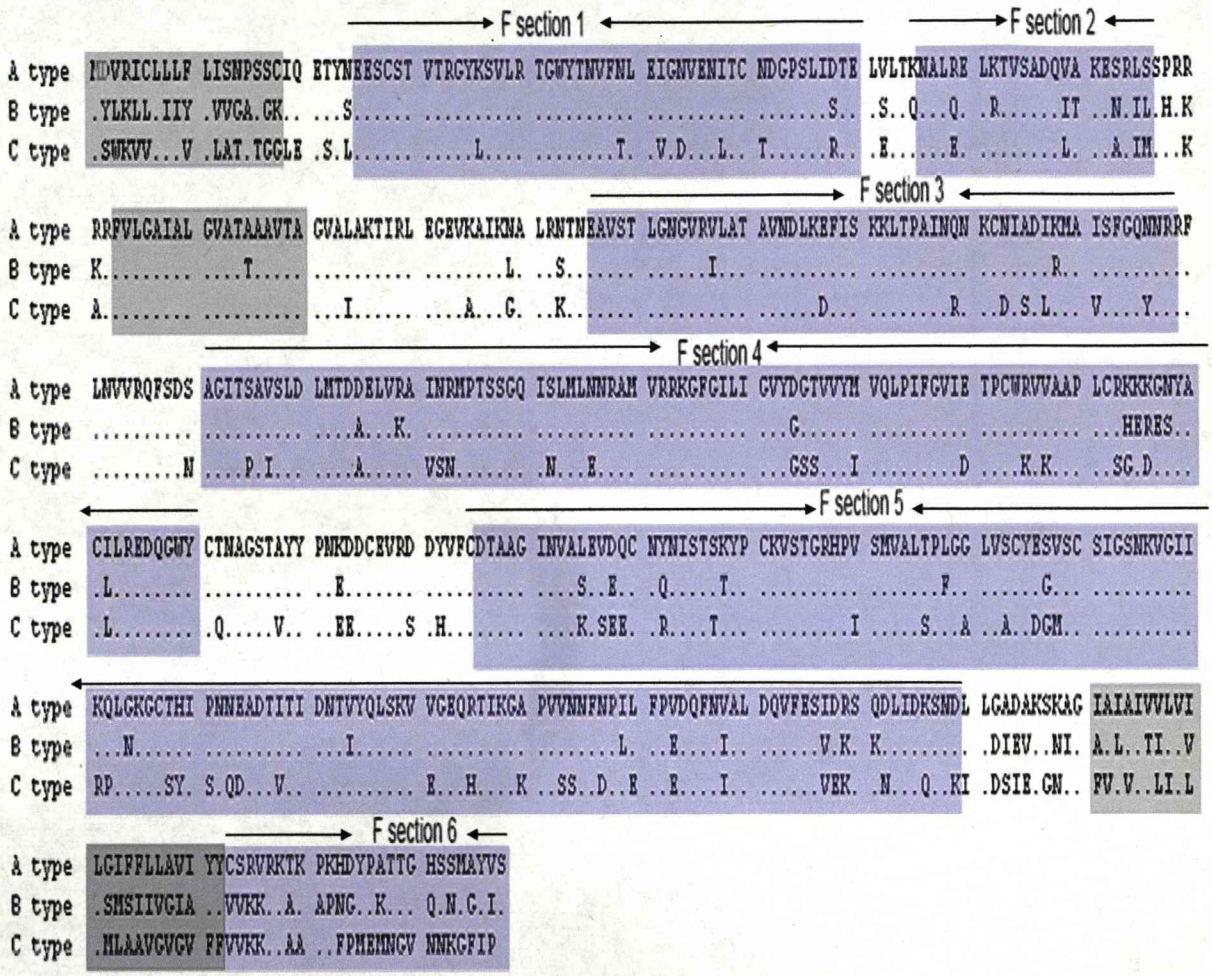


Figure 70. Predicted amino acid sequence of subtype A, B and C (A type, B type and C type) APV F protein. Six sections were designed for individual expression in *E.coli*. No sections incorporated the hydrophobic areas (Grey shaded segments). Two sections were designed in the F2 region of the F0 protein three in the F1 section and one incorporating the entire cytoplasmic tail.

6.2 Materials and methods part 1: Developing clones

6.2.1 Gene amplification

F sections 1, 3, 4 and 5 were amplified from F-p18smahis vector (developed in Chapter 4) using oligos designed to maintain the same reading frames as shown in Figure 70 and incorporate N-terminal his tags when cloned into *E.coli* expression vector p-ET30. A list of these oligos is given in table 8. Standard pfu PCR was used and the following cycle was applied: (i) 80^0C hold (hot start) (ii) 94^0C for 30 seconds (iii) 54^0C for 30 seconds (iv) 72^0C for 40 seconds (v) repeat (ii) – (iv) x 20, (vi) 8^0C hold temperature. F sections 2 and 6 were generated by annealing complementary oligos (shown in table 8) using the following PCR cycle: (i) 94^0C for 3 minutes and (ii) 40^0C > 30 minutes.

Table 8.

Gene generated Oligo Names	**Sequence 5' to 3'**
F section 1	
F2-85 pos	GAAGAATCCTGCAGTACTG
F2-223 neg	TTACTCAGTGTCAATTAGGCTGGGTCC
F section 2	
F2 no-conpep pos	GCTTTGAGGGAGCTCAAAACAGTGTCAGC TGATCAAGTGGCTAAGGAAAGCAGACTATCCTAA
F2 no-conpep neg	TTAGGATAGTCTGCTTTCCTTAGCCACTT GATCAGCTGACACTGTTTTGAGCTCCCTCAAAGC
F section 3	
F 448 pos	GAGGCAGTATCCACATTAGGG
F 610 neg	TTACCTTCTGTTATTTTGGCCAAAACTAATTGCC
F section 4	
F 634 pos	GCAGGTATCACATCAGCTGTGTCTC
F 943 neg	TTAGTACCACCCTTGATCTTCTCTCAG
F section 5	
F 1018 pos	GACACAGCAGCTGGCATTAATG
F 1450 neg	TTAGTCGTTAGACTTATCTATTAAGTCCTGAG

F section 6	
Fcyto-tailpos	TGTTCCAGAGTCCGGAAGACCAAACCAAAGCAT GATTACCCGGCCACGACAGGTCATAGCAGCATG GCTTATGTCAGTTAA
Fcyto-tailneg	TTAACTGACATAAGCCATGCTGCTATGACCTGTC GTGGCCGGGTAATCATGCTTTGGTTTGGTCTTC CGGACTCTGGAACA

6.2.2 Ligations and cloning of APV F sections with p-ET30

F sections were ligated into the EcoRV site of p-ET 30 using the Fermentas protocol but with the addition of 0.5µl (10U/µl) of EcoRV in the ligation mixture. Ligations were used to transform DH5α competent cells which were grown on LB agar plates containing 15µg/ml of Kanamycin. Resulting colonies were screened using the PCR technique described in section 4.6.2. Plasmids from colonies that were positive were extracted using Qiagen miniprep kits (3.3.5) and sent for sequencing. All products were analysed using agarose gel electrophoresis (3.3.8).

6.2.3. RE digestions

Digestions using EcoRV followed supplier's protocols.

6.3 Materials and methods part 2: Expression

6.3.1 Transformations

E.coli BL21 (DE3) cells were transformed using ~1ng of each F section/p-ET 30 plasmid construct according to the supplier's protocol (3.3.4). Colonies were grown on agar plates containing 15µg/ml of Kanamycin.

6.3.2 PCR screening

Taq PCRs were used to screen BL21 (DE3) cells that had been transformed with the above. Colonies were screened directly from agar plates as described in section 4.6.2 and Figure 27. Expected sizes for these PCRs were as follows:
F section 1 (237bp), F section 2 (420bp), F section 3 (262bp), F section 4 (400bp), F section 5 (532bp) and F section 6 (179bp).

6.3.3 Induction

Colonies that contained desired plasmids were induced as described in section 3.4.1 using 0.5mM IPTG and induction times of 1-3hours.

6.3.4 *E.coli* cell lysis

Cells were lysed as described in section 3.4.2. The efficiency of lysis was checked by gram staining (Appendix).

6.3.5 Protein purification

F section his tag recombinant proteins were purified under standard native conditions using Ni-NTA spin columns as described in section 3.4.3.

6.3.6 SDS-PAGE

Recombinant proteins were analysed under none-reducing conditions as described in section 3.6

6.3.7 Western blot

His tag western blots were used to detect purified recombinant proteins 3.6.2

6.3.8 his tag ELISAs

His tag ELISAs were performed as described in section 5.3.9 for general detection of purified recombinant proteins.

6.4 Materials and methods part 3: Development of ELISAs

6.4.1 ELISAs

Flexible, 96 well, flat bottomed ELISA plates (Becton Dickinson, UK) were used. Wells were coated with 50µl of antigen which had been previously diluted in ELISA CBC buffer (Appendix), dilutions are given along side the relevant plates in the results section. Plates were then incubated at 8^0C overnight. Antigens included his tag purified products and a Liverpool developed whole virus APV antigen. The details of where each antigen was placed in a particular ELISA, are given alongside the relevant plates in the results section.

All test antisera were hyperimmune with the exception of subtype B APV (See section 3.6.3 for details). The procedures are given below.

(i) ELISA procedures

1. Incubated plates washed 5 times with ELISA wash buffer (Appendix).
2. 50µl / well of test serum (given along side the relevant plates in the results section) previously diluted 1/100 in wash buffer, incubated for 1 hour at 37^0C.
3. Washed 5 times with wash buffer.
4. 50µl / well of GAT conjugate diluted 1/1000 in wash buffer incubated for 1 hour at 37^0C.
5. Washed 5 times with wash buffer.

6. 100µl / well of OPD activated with 30% H_2O_2 (Sigma, UK H-1009) (Appendix) incubated at room temperature in the dark for a maximum of 15 minutes.

7. 50µl / well of 2.5M H_2SO_4 (Appendix)

6.4.2 Adsorption of *E.coli* antibodies from test serum

E.coli antibodies were removed from test serum using the techniques illustrated in Figure 71. These were adapted from Islam 1988 [222]

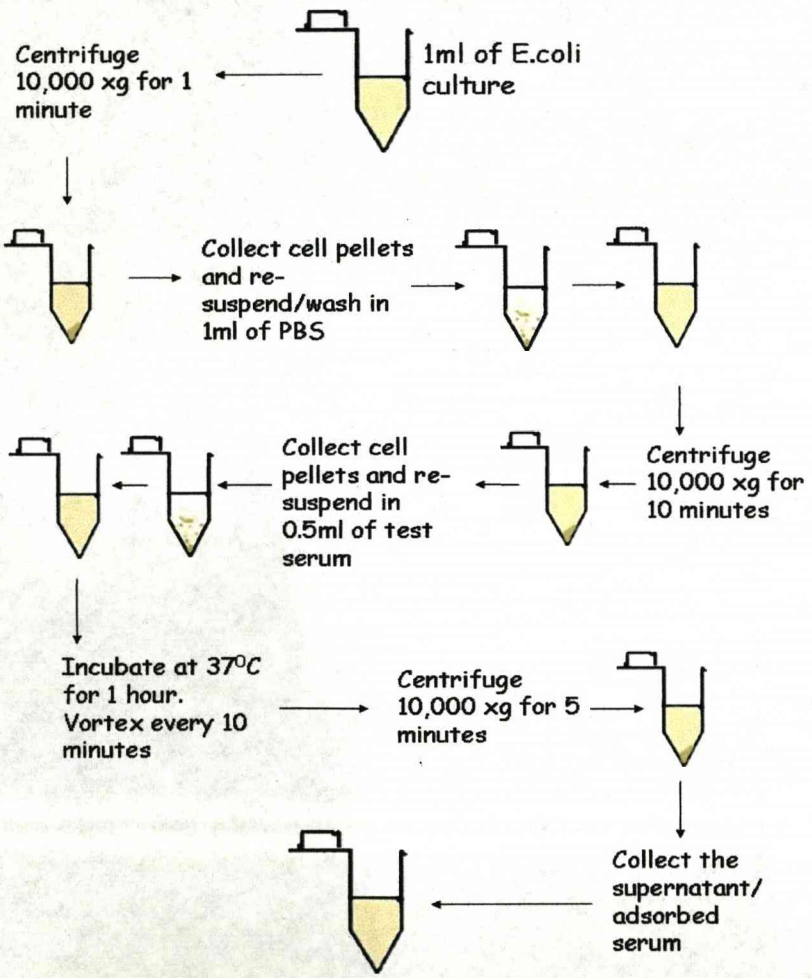


Figure 71. Adsorption of *E.coli* antibodies from serum

6.4.3 Agar gel precipitation (AGP)

AGP was used to analyse the efficiency of *E.coli* antibody adsorption from test serum. In these tests both antigen and antibody diffuse through an agarose matrix and precipitate at equivalence points. Lines of precipitation can then be observed using a dark background and oblique illumination. 10 ml of molten agar (1% in PBS) was poured into a plastic Petri dish and allowed to solidify at room temperature. Wells were cut in each plate using a template which produced one center well surrounded by six outer wells in a daisy pattern. Wells were 7mm in diameter and 4mm apart. Plugs were carefully removed using a hypodermic needle. Lysed *E.coli* cells (antigen) were placed into the centre well and test sera were placed in surrounding wells. Plates were placed into small sealable 16mm x 10mm polythene bags and incubated at 37^0C for 24 hours. Details of test sera used in each particular AGP is shown in the relevant results section.

6.4.4 IP staining

In common with ELISAs and western blot, IP staining demonstrates antigen-antibody interactions. It is used to detect antigens in microscopic preparations (smears, tissue sections, cell cultures). The anti-globulins are tagged with an enzyme which, following the application of a chromogen substrate, produce a detectable colour in positive areas.

Here, IP staining of uninfected and APV infected vero cell sheets (grown in 48 well cell culture plates) was used to test for APV specific antibodies in SPF serum of 12 week old (12wk) and 80 week old (80wk) chickens. An APV positive serum was used as a control. The protocol used was as follows:

(i) Remove medium form cell sheets with pastettes

(ii) Fix in 70% ethanol for 10 minutes (~ 500µl / well)

(iii) Wash gently x 3 with PBS

(iv) Add 300µl of test serum (diluted 1/500 in PBS) to each well and incubate for 1 hour at 37^{0}C (Figure 72 details each test serum)

(v) Wash gently x 3 with PBS

(vi) Add 300µl of GAT conjugate (diluted 1/500 in PBS) to each well and incubate for 1 hour at 37^{0}C

(vii) Wash gently x 3 with PBS and x1 with DAB substrate buffer (Appendix)

(viii) Add 300µl of DAB substrate solution (Appendix) to each well and incubated in the dark.

(viiii) Stop reaction by x 4 gentle washes with PBS.

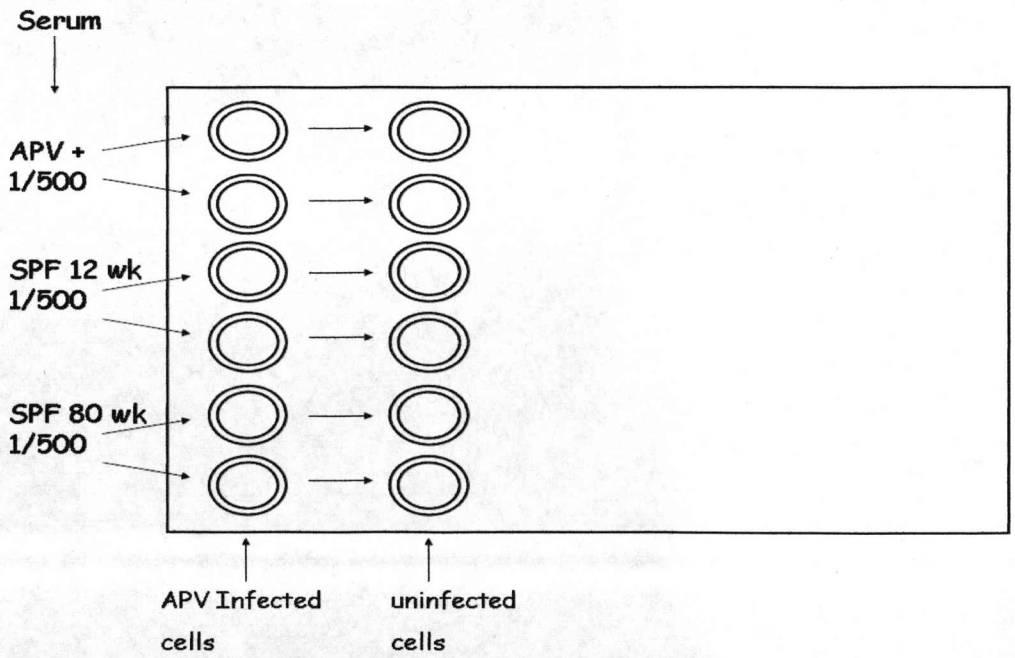


Figure 72. IP staining of APV infected and uninfected vero cell sheets using APV + and SPF serum from 12wk old and 80wk old birds.

6.5 Materials and methods part 4: Virus neutralization

A two part, virus neutralization test was designed to identify potential neutralizing epitopes within the expressed regions of the fusion protein:

1. Standard neutralization tests were performed in vero cells using a SH-G deletion recombinant, subtype-A APV [72] and subtype A, B and C antiserum to identify end point dilutions. Use of SH-G deletion recombinant (ΔSH-G) APV eliminated any virus neutralizing properties of antibodies previously shown to target the G protein [91].

2. Neutralization test were then repeated following adsorption of the same Subtype A, B and C antiserum with each of the antigenic F sections. Thus, if neutralizing antibodies were absorbed by a particular F antigen then endpoints at lower dilutions would be expected. Those with two $\log_2$ dilutions or greater compared to the control value, would be considered significant. Therefore, the relative contributions of the different regions of the F protein in eliciting neutralizing antibodies could be assessed.

6.5.1 Standard virus neutralization test

50µl of tissue culture media without serum (appendix) was added / well of an 8 X 12, 96-well tissue culture plate (NUNC international Denmark). This was followed by 50µl of pooled, APV subtype-A, B or C positive anti sera (previously diluted to 1/16) to the first column of wells (specific placement of each antiserum is given along side the results). Next a series of doubling dilutions were made up to column 10 giving a $\text{Log2}^{5\text{-}14}$ range of dilutions. 50µl of ΔSH-G virus suspension at a Log 10^2 /50µl concentration was then added and thoroughly mix to columns 1-11 leaving column 12 as a cell control. Plates were then incubated at 37^0C for 3 hours. Each 100µl suspension was transferred to a corresponding well

containing a fully confluent vero-cell monolayer. Following a second 3hr incubation at 37^0C each well received 50µl of tissue culture medium containing serum (appendix). CPE was examined daily using light microscopy.

6.5.2 Novel virus neutralization test

25µl of tissue culture media without serum (appendix) was added / well of an 8 X 12, 96-well tissue culture plate (NUNC international Denmark). This was followed by 25µl of pooled, APV subtype-A, B or C positive anti sera (previously diluted to 1/64 for subtype A and B and 1/16 for subtype C) to the first column of wells (specific placement of each anti sera is give in the results). Next a series of doubling dilutions were made up to column 8. Tests using subtype-A and B antisera had a $Log2^{7-14}$ range of dilutions with subtype C range at $Log2^{5-12}$. Next 25µl of F antigen 1, 2, 3, 4, 5, 6 or control Ni-NTA elution buffer (appendix) plus glycerol at the same concentration as purified F antigens (previously diluted 1/50 in tissue culture medium) was added. Again details of their position in a particular test are given in the relevant results section. Plates were incubated for 3hrs. 25µl of ΔSH-G virus suspension at a Log 10^2 /50µl concentration was then added to columns 1-11 leaving column 12 as a cell control. Plates were again incubated at 37^0C for 3 hours. Each 100µl suspension was transferred to a corresponding well containing a fully confluent vero-cell monolayer. Following a final, 3hr incubation at 37^0C each well received 75µl of tissue culture medium containing serum (appendix).

CPE was examined daily using light microscopy.

6.6 Results part 1: Developing clones

Figure 73 shows F sections 1, 3, 4 and 5 that had been amplified from F-p18smahis. Each band correlated with the calculated size of each f section (F section 1 = 138bp, F section 3 = 162bp, F section 4 = 309bp and F section 5 = 432bp.

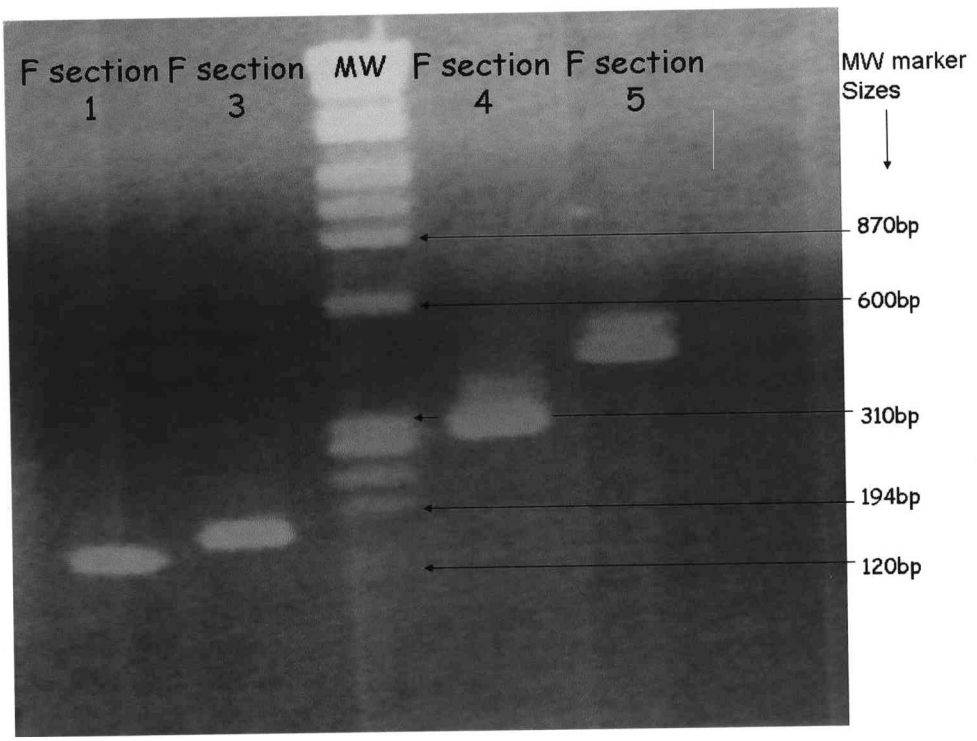


Figure 73. Pfu PCR amplification of F sections 1, 3, 4 and 5 (agarose gel stained with ethidium bromide)

F sections 2 and 6 were not analysed prior to ligation with p-ET30.

All six F sections were cloned into p-ET30. Sequences of these constructs are shown in Figures 74-79.

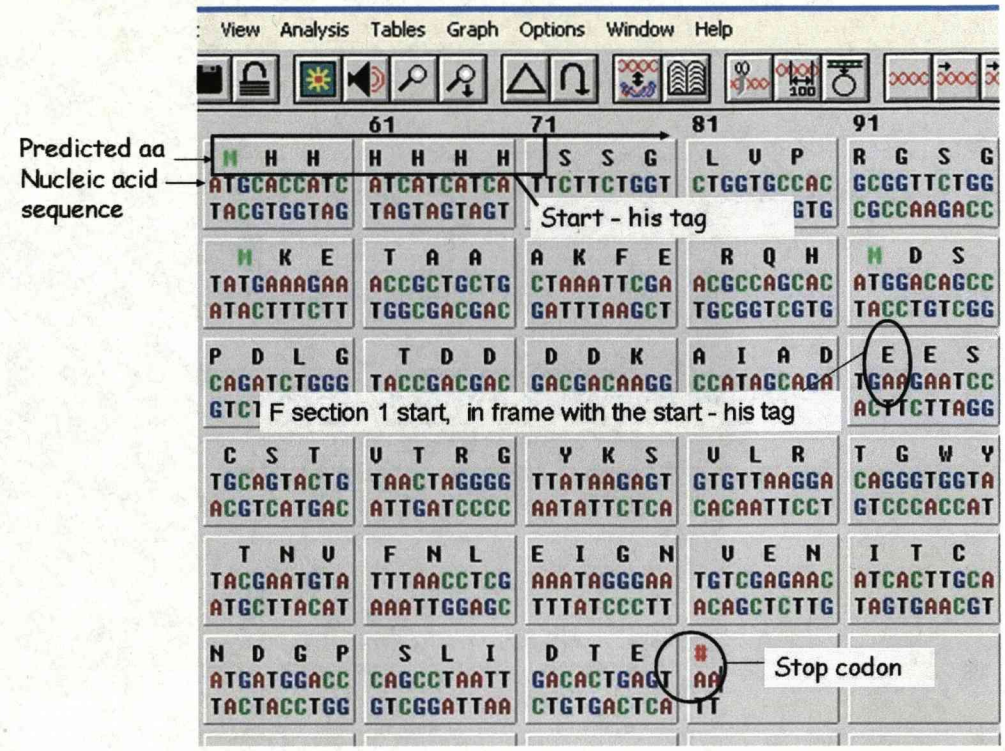


Figure 74. Nucleic and predicted amino acid sequence of F section 1 in p-ET 30 with an N terminal his tag region

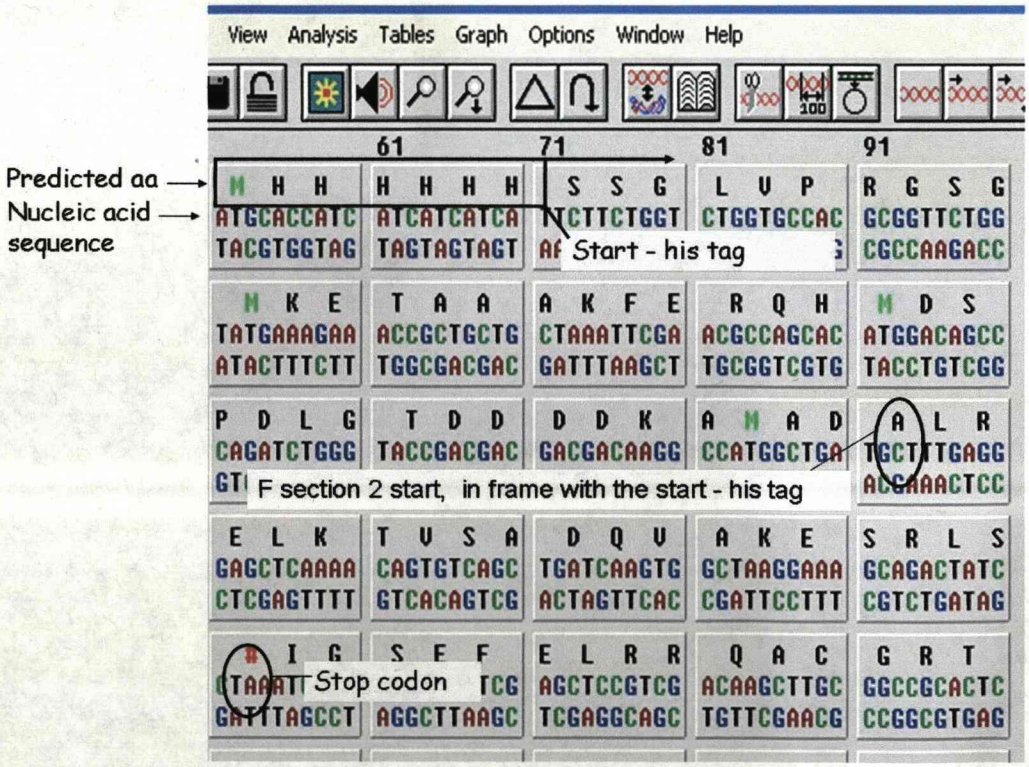


Figure 75. Nucleic and predicted amino acid sequence of F section 2 in p- ET 30 with an N terminal his tag region

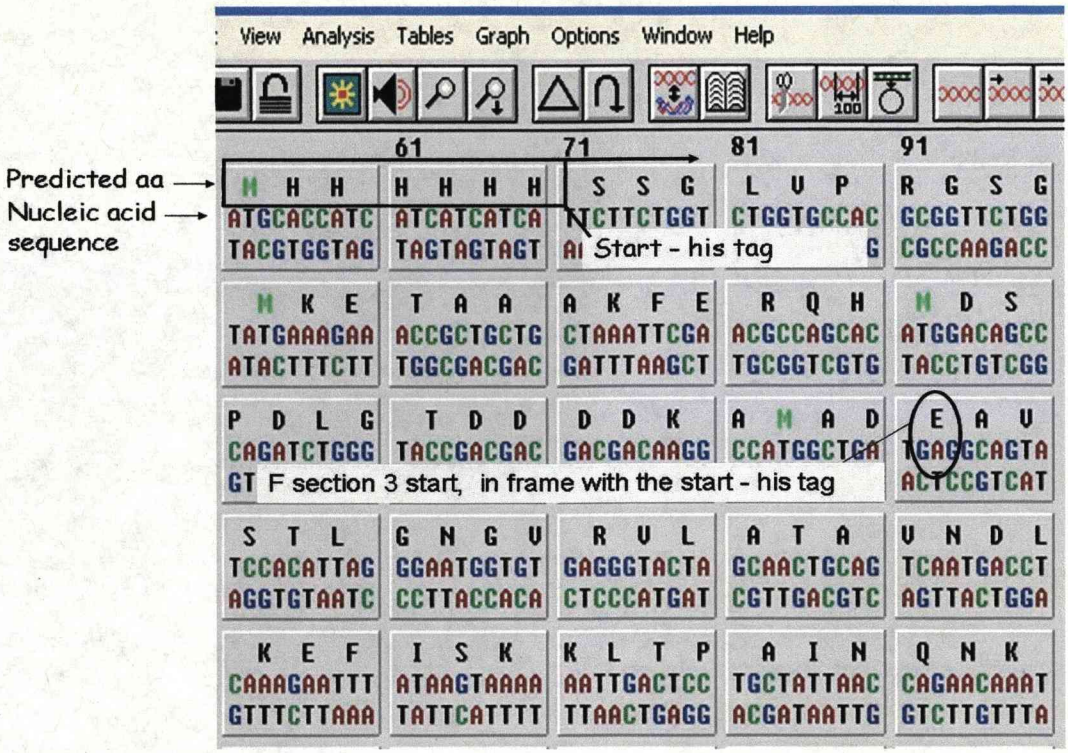


Figure 76. Nucleic and predicted amino acid sequence of F section 3 in p-ET 30 with an N terminal his tag region

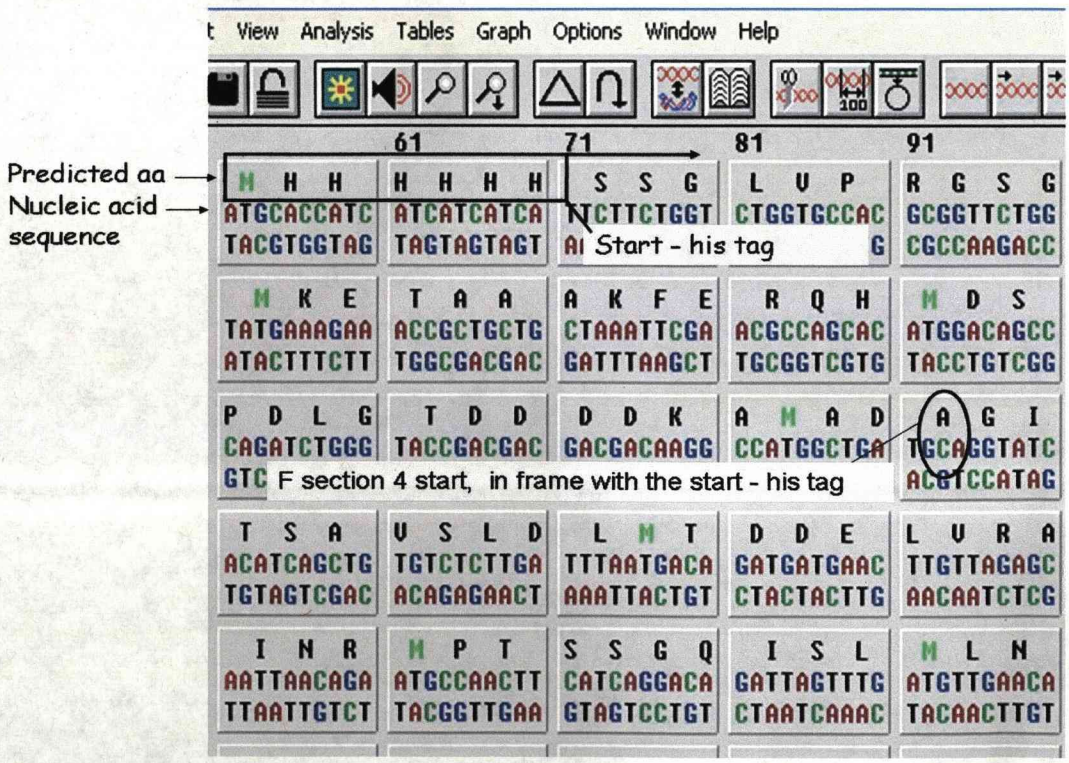


Figure 77. Nucleic and predicted amino acid sequence of F section 4 in p- ET 30 with an N terminal his tag region

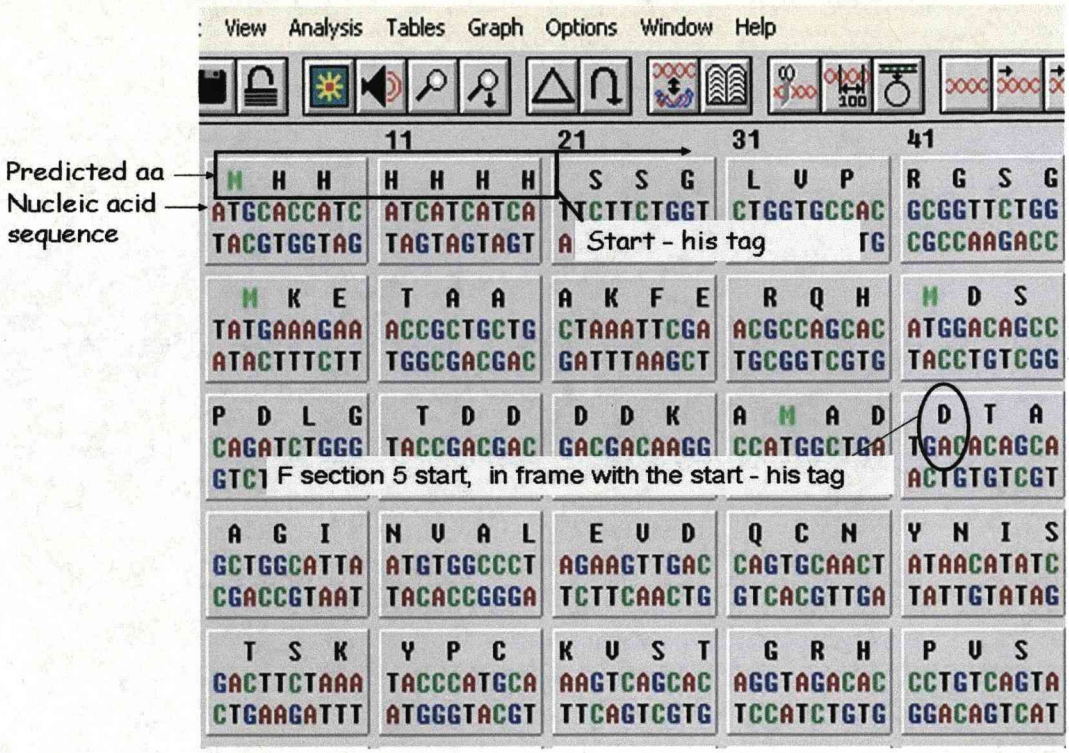


Figure 78. Nucleic and predicted amino acid sequence of F section 5 in p- ET 30 with an N terminal his tag region

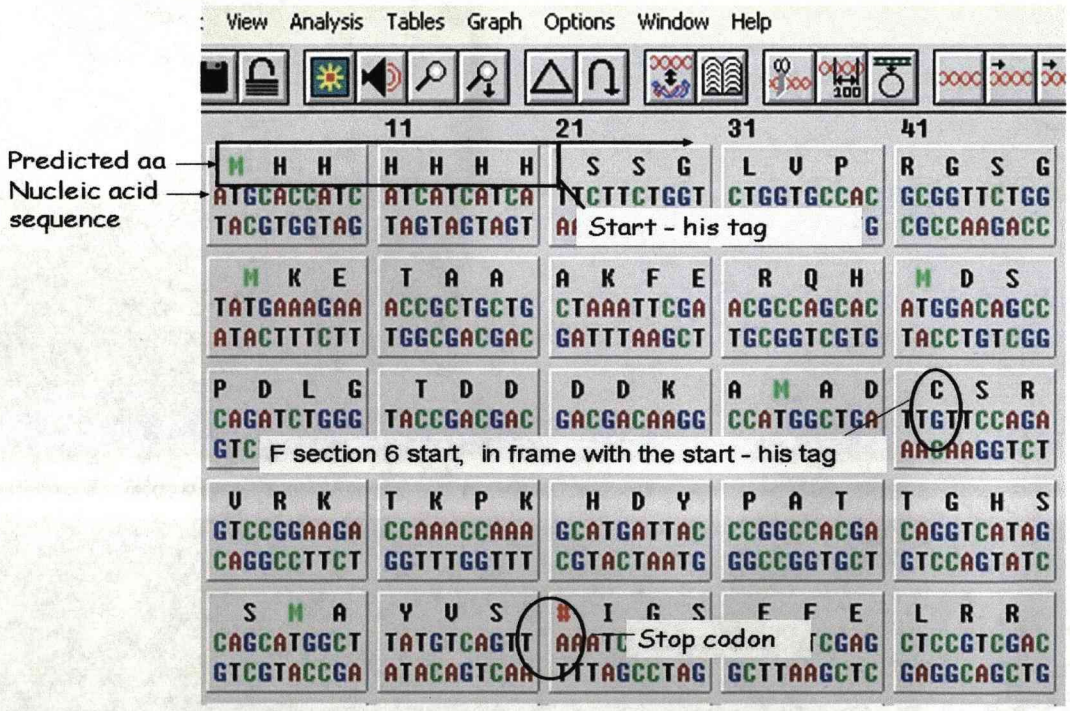


Figure 79. Nucleic and predicted amino acid sequence of F section 6 in p- ET 30 with an N terminal his tag region

F sections 1, 2 and 6 can be seen in their entirety (from start to stop codon) in figures 74, 75 and 79. The subsequent sections of 3, 4 and 5 remained in frame to the designed stop codons. Each predicted aa sequence correlated with those highlighted in Figure 70.

6.7 Results part 2: Expression

6.7.1 PCR screening

100% of screened BL21 (DE3) *E.coli* cells, yielded clones of each F section that correlated with expected sizes (section 6.3.2). These were identified using the Taq PCR method (4.6.2). Products are shown in Figure 80.

Figure 80. PCR screen of BL21 (DE3) *E.coli* colonies. A, B and C represent individual colonies of the particular F section listed above it (agarose gel stained with ethidium bromide)

6.7.2 *E.coli* cell lysis

Lysis of F section clones induced for 1-3hours was as demonstrated by gram stain (Figure 81).

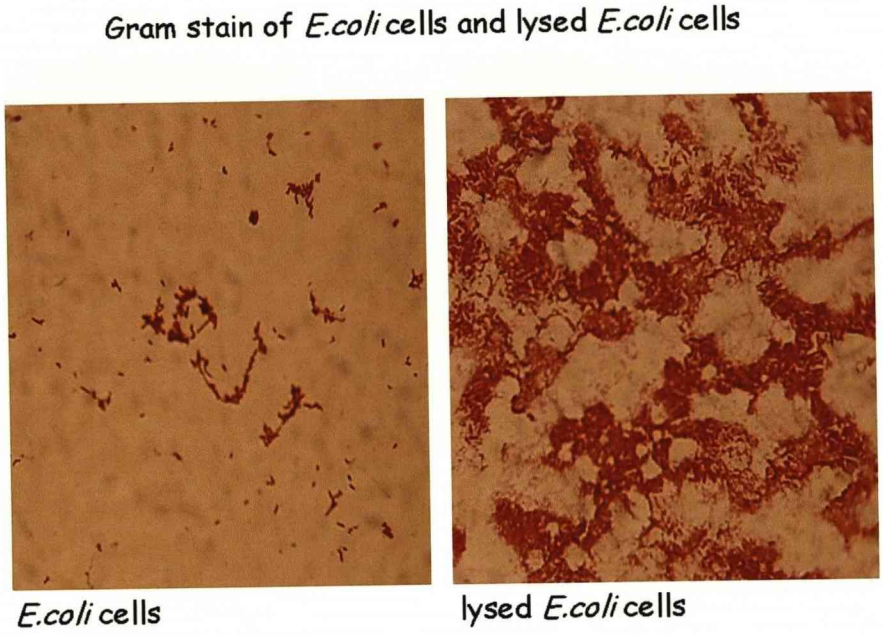


Figure 81. Analysis of *E.coli* cell lysis using gram staining

6.7.3 his tag ELISAs

His tag ELISAs demonstrated recombinant F section proteins in purified samples following native his tag purification as shown in Figure 82. An increase in protein yield was observed over the three hour induction period for both A and B clones of each F section. F section 2 gave the weakest signal and was only detectable in the sample purified from a culture taken at 3 hours post induction in this ELISA.

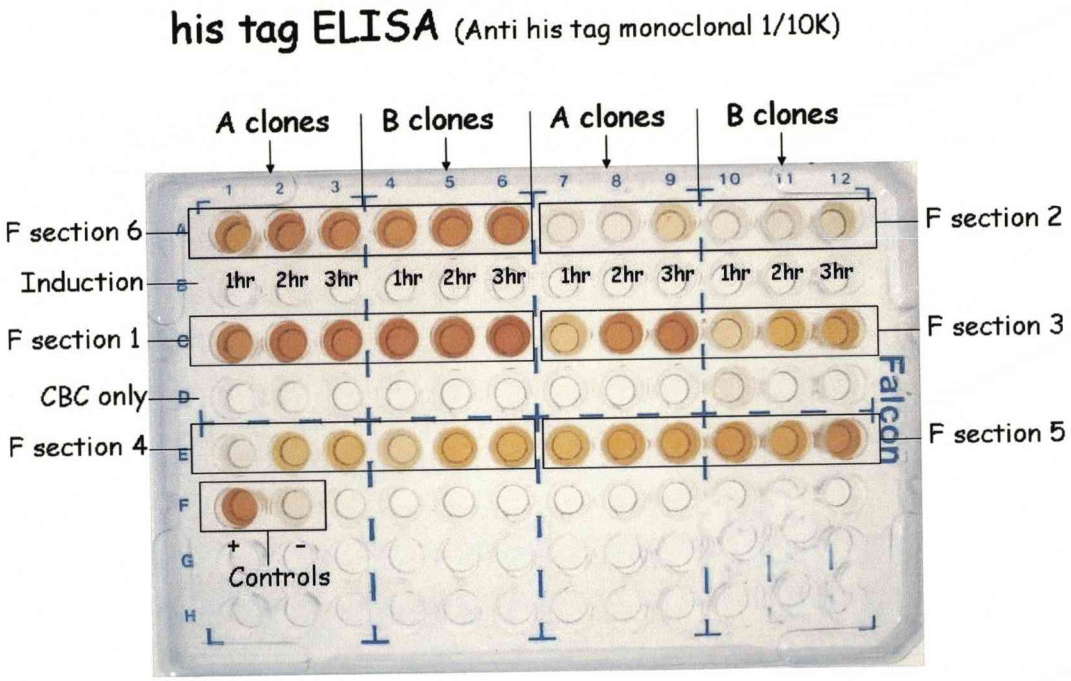


Figure 82. his tagged, recombinant F section proteins, detected in purified samples following native his tag purification. The his tag epitope of recombinant proteins was detected in this ELISA using an anti his tag monoclonal antibody.

6.7.4 SDS-PAGE and Western blot analysis

F section samples purified after 3 hours induction, and positive by his tag ELISA, (Figure 82) were analysed by SDS-PAGE and his tag Western blot. SDS-PAGE analysis of F sections 2 and 6 produced strong bands of approximately 11.6KDa and 10KDa respectively. F section 5 produced a weak band of approximately 25KDa and F sections 1, 3 and 4 were undetectable as shown in Figure 83. However, when tested in a his tag western blot as shown in Figure 84, all were positive with the exception of F section 2. A standard curve (Figure 85) generated in the same way as described in 4.7.6 was used to determine the band sizes shown in Figure 83. F section 1 gave a band of 13 KDa, F section 3; 12.5 KDa, F section 4; 15 KDa, F section 5; 25KDa and F section 6; 11 KDa. All

absorbance were close to expected sizes however; F section 3 gave a band that was 2KDa smaller than expected.

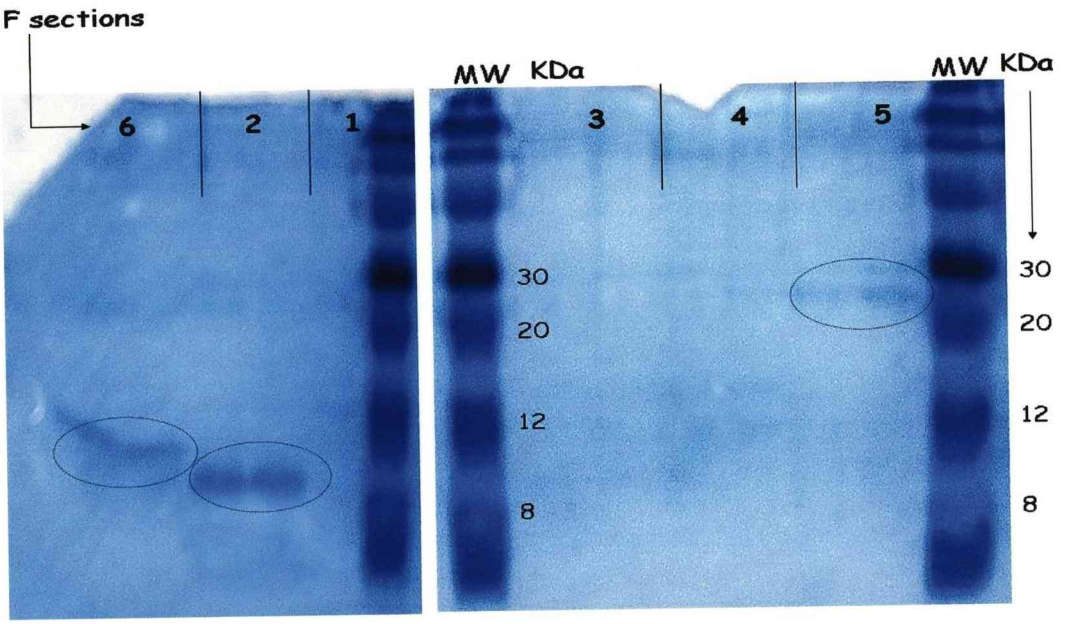


Figure 83. SDS-PAGE analysis of his tag purified F sections.

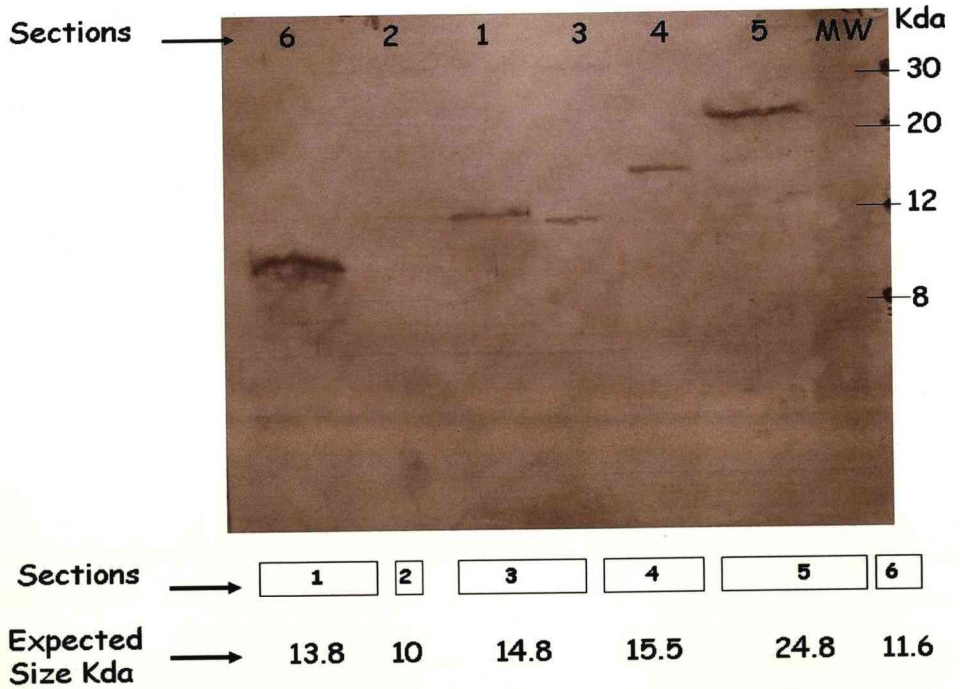


Figure 84. his tag Western blot of expressed and his tag purified F sections

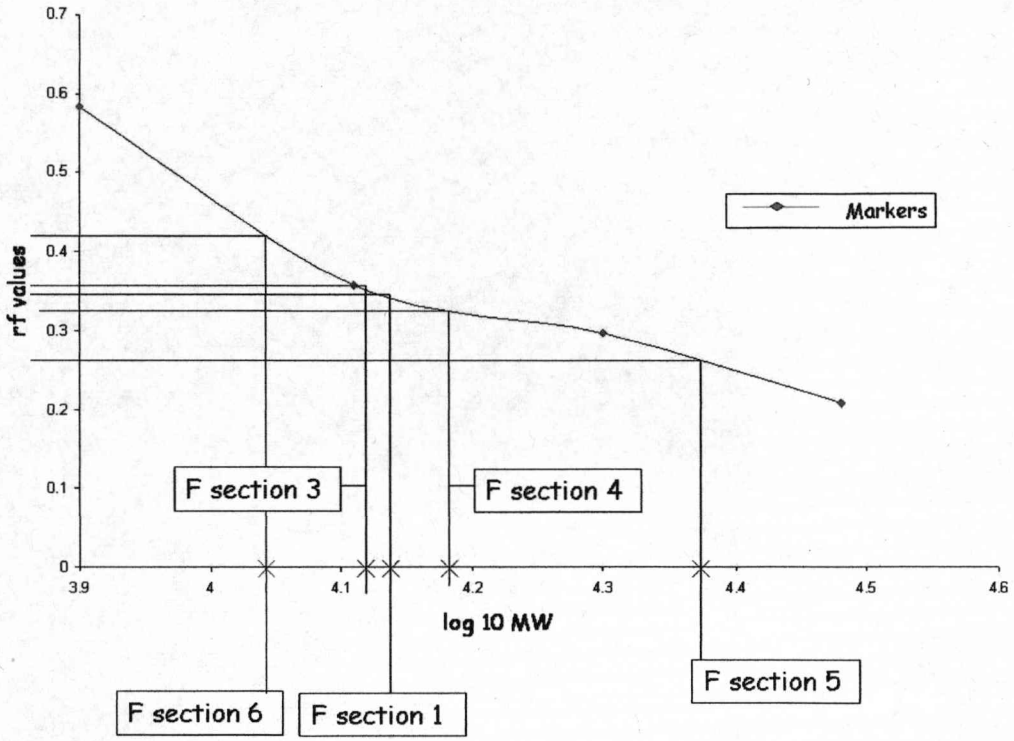


Figure 85. Log_{10} MW of F sections 1, 4, 5 and 6 following Western blot analysis

6.8 Results part 3: Development of ELISAs

The samples tested by his tag ELISA, SDS-PAGE and Western blot (Figures 82, 83 and 84) were used in APV ELISAs. Each was tested alongside a Liverpool developed whole virus subtype-A APV antigen with SPF serum from 12 wk old chickens as controls. Sections were tested at 1/50 and 1/100 dilution with subtype-A antiserum producing the ELISA shown in Figure 86. In view of their antigenicity, F sections were referred to as F antigens from this time. Subsequently, antigens were tested with subtype A, B and C APV positive serum and with two negative controls (i) IBV positive serum and (ii) SPF serum from 12 wk old chickens (6.7.1)

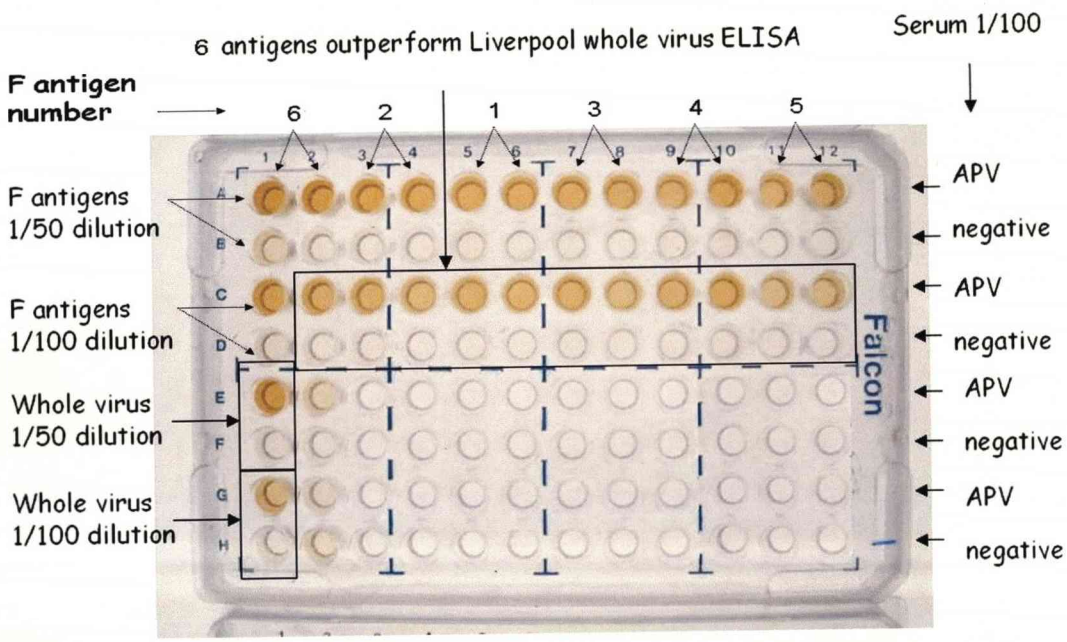


Figure 86. F antigens tested with subtype-A antiserum.

Absorbances were measured at 492nm. Absorbances for the test serum were divided by negative readings to give the ratios shown in Table 9. Ratios showed

that at 1/100 dilution, all six F sections produced higher absorbance ratios than the Liverpool whole virus antigen.

Table 9. Absorbencies at 492nm

F Ag	6	2	2	1	1	3	3	4	4	5	5	Virus Ag
APV pos serum	1.2	1.1	0.9	1.1	1	1.1	0.9	1.1	1	0.8	0.8	0.8
SPF serum 12 wk	0.1	0.09	0.07	0.08	0.07	0.07	0.06	0.1	0.1	0.07	0.07	0.1
ratio	12	12.2	12.9	13.8	14.3	15.7	15	11	10	11.4	11.4	8

Following these results F antigens were stored at -20^{0}C for subsequent ELISA tests (6.7.1) however, after thawing, precipitates had formed. As a result, new batches of purified F antigens were generated (as previously described) and each was mixed at a ratio of 50:50 with glycerol to maintain their solubility. Because glycerol may be detrimental to antigen performance however; ELISAs were repeated and results were found to be similar to those found previously (Figure 87). Glycerol was added to all recombinant F antigens from this time.

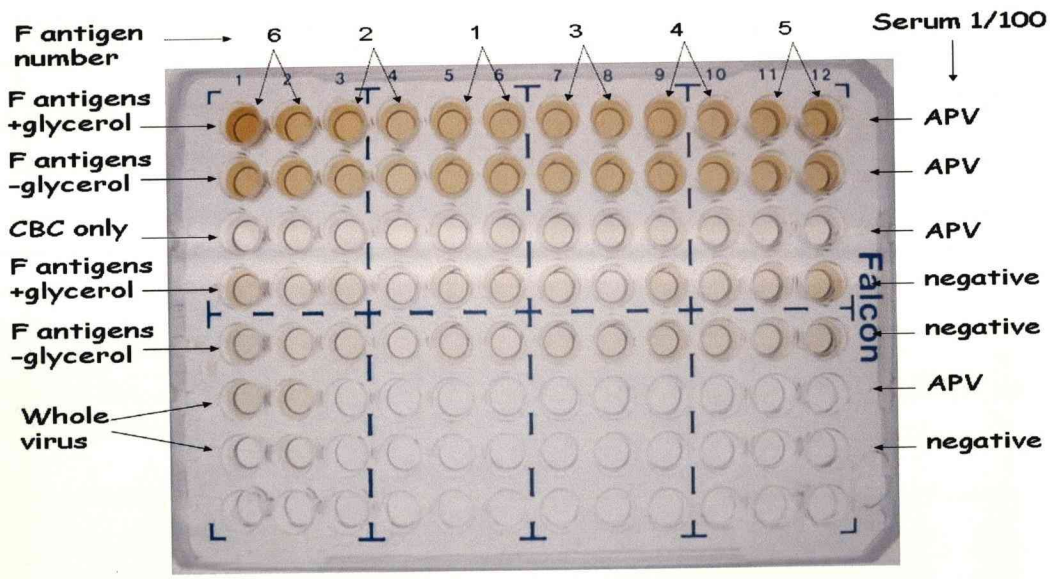


Figure 87. F antigens with and without 50% glycerol, tested with subtype-A antisera.

6.8.1 F antigens tested against subtype A, B and C antisera

ELISAs testing each F antigen and subtype-A APV whole virus antigen against six pools (10 birds / pool) of positive subtype A, B or C chicken antisera, IBV positive antisera and SPF sera are given below. Each serum, F antigen and whole virus antigen was used at a 1/100 dilution. Tables 10-15 show duplicate and mean absorbance readings at 492nm for each F antigen. Tables 16-18 show duplicate and mean absorbance readings at 492nm for whole virus antigen.

Table 10. Subtype-A antiserum with F antigens 1 - 3

A + sera									
	F Ag1	F Ag 1	Mean	F Ag 2	F Ag 2	Mean	F Ag 3	F Ag 3	Mean
pool									
1	0.75	0.53	**0.64**	0.47	0.5	**0.485**	0.54	0.53	**0.54**
2	0.98	0.9	**0.94**	0.84	0.82	**0.83**	1	1.1	**1.05**
3	0.71	0.63	**0.67**	0.6	0.6	**0.6**	0.68	0.67	**0.68**
4	1.02	1.04	**1.03**	0.94	0.95	**0.945**	1.51	1.45	**1.48**
5	1.95	1.8	**1.87**	1.6	1.6	**1.6**	2.29	2.2	**2.25**
6	0.84	0.76	**0.8**	0.71	0.67	**0.69**	1.03	1.04	**1.04**
spf	0.18	0.12	**0.15**	0.12	0.12	**0.12**	0.12	0.12	**0.12**
IBV	0.15	0.17	**0.16**	0.13	0.12	**0.12**	0.19	0.15	**0.17**

Table 11. Subtype-A antiserum with F antigens 4 - 6

A + sera									
	F Ag 4	F Ag 4	Mean	F Ag 5	F Ag 5	Mean	F Ag 6	F Ag 6	Mean
pool									
1	0.62	0.6	**0.61**	0.63	0.6	**0.62**	0.68	0.9	**0.79**
2	1.1	1.1	**1.1**	1.1	0.98	**1.04**	1	1.2	**1.1**
3	1.3	1.2	**1.25**	1.1	1	**1.05**	0.78	0.93	**0.86**
4	1.5	1.5	**1.5**	1.5	1.4	**1.45**	1.7	1.9	**1.8**
5	2.2	2.2	**2.2**	2.3	2.1	**2.2**	2	2.03	**2.02**
6	1.02	0.96	**0.99**	1	0.96	**0.98**	1.04	1.04	**1.04**
spf	0.14	0.14	**0.14**	0.14	0.16	**0.15**	0.15	0.3	**0.23**
IBV	0.18	0.12	**0.15**	0.13	0.15	**0.14**	0.18	0.18	**0.18**

Table 12. Subtype-B antiserum with F antigens 1 - 3

B + sera									
	F Ag1	F Ag 1	Mean	F Ag 2	F Ag 2	Mean	F Ag 3	F Ag 3	Mean
pool									
1	0.2	0.12	**0.16**	0.11	0.11	**0.11**	0.12	0.12	**0.12**
2	0.71	0.66	**0.68**	0.62	0.62	**0.62**	0.78	0.76	**0.78**
3	0.53	0.43	**0.48**	0.41	0.4	**0.41**	0.48	0.45	**0.47**
4	0.23	0.21	**0.22**	0.18	0.17	**0.18**	0.21	0.21	**0.21**
5	0.32	0.29	**0.31**	0.29	0.27	**0.28**	0.35	0.33	**0.34**
6	0.147	0.117	**0.13**	0.1	0.1	**0.1**	0.11	0.11	**0.11**
spf	0.12	0.08	**0.1**	0.06	0.07	**0.07**	0.08	0.07	**0.08**
IBV	0.1	0.12	**0.11**	0.13	0.13	**0.13**	0.12	0.15	**0.14**

Table 13. Subtype-B antiserum with F antigens 4 - 6

B + sera									
	F Ag 4	F Ag 4	Mean	F Ag 5	F Ag 5	Mean	F Ag 6	F Ag 6	Mean
pool									
1	0.18	0.17	**0.18**	0.16	0.17	**0.17**	0.13	0.25	**0.19**
2	0.95	0.95	**0.95**	0.85	0.85	**0.85**	0.69	0.78	**0.74**
3	0.72	0.67	**0.70**	0.58	0.63	**0.61**	0.5	0.64	**0.57**
4	0.72	0.7	**0.71**	0.47	0.43	**0.45**	0.24	0.33	**0.29**
5	0.38	0.37	**0.38**	0.28	0.35	**0.32**	0.32	0.32	**0.32**
6	0.14	0.15	**0.15**	0.15	0.13	**0.14**	0.14	0.27	**0.21**
spf	0.12	0.08	**0.1**	0.06	0.07	**0.07**	0.08	0.07	**0.08**
IBV	0.18	0.13	**0.16**	0.2	0.14	**0.17**	0.12	0.16	**0.14**

Table 14. Subtype-C antiserum with F antigens 1 - 3

C + sera									
	F Ag1	F Ag 1	Mean	F Ag 2	F Ag 2	Mean	F Ag 3	F Ag 3	Mean
pool									
1	1.76	1.7	**1.73**	1.58	1.6	**1.59**	2	2.1	**2.05**
2	2	1.9	**1.95**	1.75	1.85	**1.8**	2.3	2.3	**2.3**
3	1.7	1.74	**1.72**	1.71	1.49	**1.6**	2.1	2.02	**2.06**
spf	0.15	0.11	**0.13**	0.09	0.085	**0.09**	0.11	0.1	**0.10**
IBV	0.13	0.13	**0.13**	0.16	0.19	**0.18**	0.11	0.15	**0.13**

Table 15. Subtype-C antiserum with F antigens 4 - 6

C + sera									
	F Ag 4	F Ag 4	Mean	F Ag 5	F Ag 5	Mean	F Ag 6	F Ag 6	Mean
pool									
1	2.2	2.3	**2.25**	2.2	2	**2.1**	1.7	1.7	**1.7**
2	2.5	2.5	**2.5**	2.3	2.3	**2.3**	1.8	1.8	**1.8**
3	2.3	2.1	**2.2**	2.1	2.11	**2.12**	1.6	1.6	**1.6**
spf	0.1	0.13	**0.12**	0.12	0.11	**0.12**	0.13	0.1	**0.12**
IBV	0.17	0.12	**0.15**	0.13	0.11	**0.12**	0.15	0.16	**0.16**

Table 16. Subtype-A antiserum with subtype-A whole virus antigen

A+ sera			
	Liverpool whole virus antigen		
pool			mean
1	0.63	0.6	**0.66**
2	0.73	0.68	**0.71**
3	0.72	0.7	**0.71**
4	0.53	0.58	**0.56**
5	0.55	0.52	**0.54**
6	0.52	0.49	**0.51**
spf	0.18	0.1	**0.14**
IBV	0.08	0.1	**0.9**

Table 17. Subtype-B antiserum with subtype-A whole virus antigen

B+ sera			
	Liverpool whole virus antigen		
pool			mean
1	0.3	0.31	**0.31**
2	0.41	0.39	**0.4**
3	0.32	0.4	**0.36**
4	0.21	0.23	**0.22**
5	0.2	0.2	**0.2**
6	0.14	0.18	**0.16**
spf	0.2	0.1	**0.15**
IBV	0.12	0.1	**0.11**

Table 18. Subtype-C antiserum with subtype-A whole virus antigen

C+ sera			
	Liverpool whole virus antigen		
pool			**mean**
1	0.39	0.33	**0.36**
2	0.3	0.28	**0.29**
3	0.4	0.32	**0.36**
spf	0.17	0.15	**0.16**
IBV	0.16	0.09	**0.13**

Table 19 summarizes the above data providing an average of the given mean results minus SPF negative control readings for each F antigen and whole virus antigen, with each particular APV antiserum. All six F antigens gave higher absorbance readings than whole virus antigen with all antisera but especially C.

Table 19.

	Subtype A fusion protein sections						**Subtype A Whole virus**
	F Ag 1	**F Ag 2**	**F Ag 3**	**F Ag 4**	**F Ag 5**	**F Ag 6**	
	Mean	**Mean**	**Mean**	**Mean**	**Mean**	**Mean**	**Mean**
Subtype A anti sera	0.84	0.74	1.05	1.15	1.07	1.05	0.46
Subtype B anti sera	0.23	0.22	0.26	0.42	0.35	0.25	0.12
Subtype C anti sera	1.67	1.6	2.03	2.2	2.05	1.58	0.17

Relative antigenicities were calculated as percentages of the highest recording (antigen 4) with subtype A and B antisera these are shown in Tables 20-21. Table 22 shows amino acid homologies for each F antigen between subtypes A, B and C.

Table 20. Relative antigenicity of F antigens 1, 2, 3, 5 and 6 to antigen 4 with subtype A antiserum

	F Ag 1	F Ag 2	F Ag 3	F Ag 4	F Ag 5	F Ag 6
	Mean	Mean	Mean	Mean	Mean	Mean
Subtype A antisera	0.85	0.74	1.05	1.15	1.07	1.05
Relative % toAg4	74	67	92	100	93	91

Table 21. Relative antigenicity and of F antigens 1, 2, 3, 5 and 6 to antigen 4 with subtype B antiserum

	F Ag 1	F Ag 2	F Ag 3	F Ag 4	F Ag 5	F Ag 6
	Mean	Mean	Mean	Mean	Mean	Mean
Subtype B antisera	0.23	0.22	0.26	0.42	0.35	0.25
Relative % toAg4	55	52	61	100	83	60

Table 22. Amino acid homologies of F antigens, between Subtypes A, B and C

Subtype A F antigen	1	2	3	4	5	6
% amino acid homology with Subtype B	98	65	96	91	90	46
% amino acid homology with Subtype C	87	85	87	84	78	14

At this time, F antigens were sent to Biochek, ltd (Hounslow, UK) for commercial APV ELISA analysis. Initial testing produced desired results but when further tested against SPF serum from 80wk old chickens all produced very high background absorbances. Similar testing of the serum in Liverpool produced the similar high absorbencies when compared to absorbencies of SPF serum from 12 wk old chickens. Moreover, absorbencies were higher than those for APV positive serum against three F antigens, namely 1, 2 and 6 as show in Table 23.

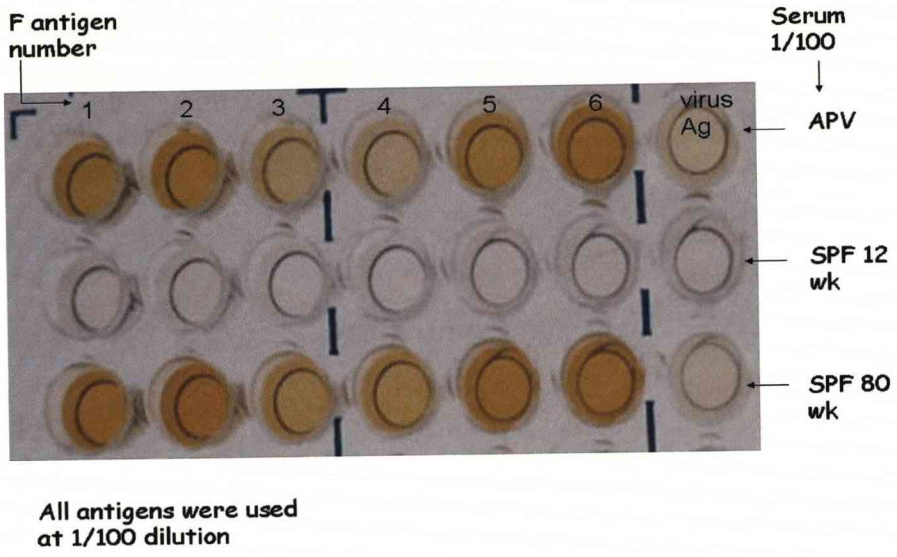


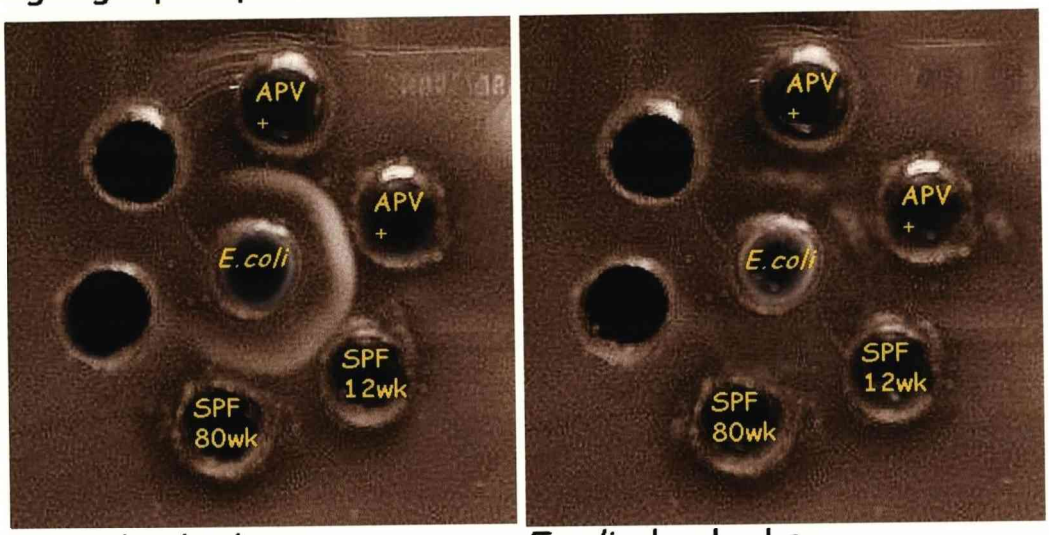
Figure 88. APV ELISA of F antigens and an APV whole virus control (virus Ag). SPF 80wk serum gives high background.

Table 23. Absorbencies at 492nm

F Ag	1	2	3	4	5	6	virus Ag
APV pos serum	1.6	1.7	1	1	1	1.3	0.6
SPF serum (12wk)	0.05	0.08	0.03	0.03	0.03	0.03	0.04
SPF serum (80wk)	1.9	1.8	0.8	0.7	1	1.5	0.2

To determine whether unwanted, interactions between specific *E.coli* antibody and E coli antigen in the antigen preparations were the cause, the SPF (80wk) serum was pre-adsorbed using an *E.coli* extract. APV positive and SPF 12 wk serum were also pre-adsorbed. The absorbed sera were assessed by AGP as shown in Figure 89. Subsequently adsorbed and non adsorbed sera were tested in an APV ELISA (Figure 90)

Agar gel precipitation test



Non adsorbed sera *E.coli* adsorbed sera

Serum: APV +, SPF 12wk and SPF 80wk

Antigen: *E.coli*

Figure 89. AGP of non adsorbed and *E.coli* adsorbed sera using lysed E.coli cells as antigen.

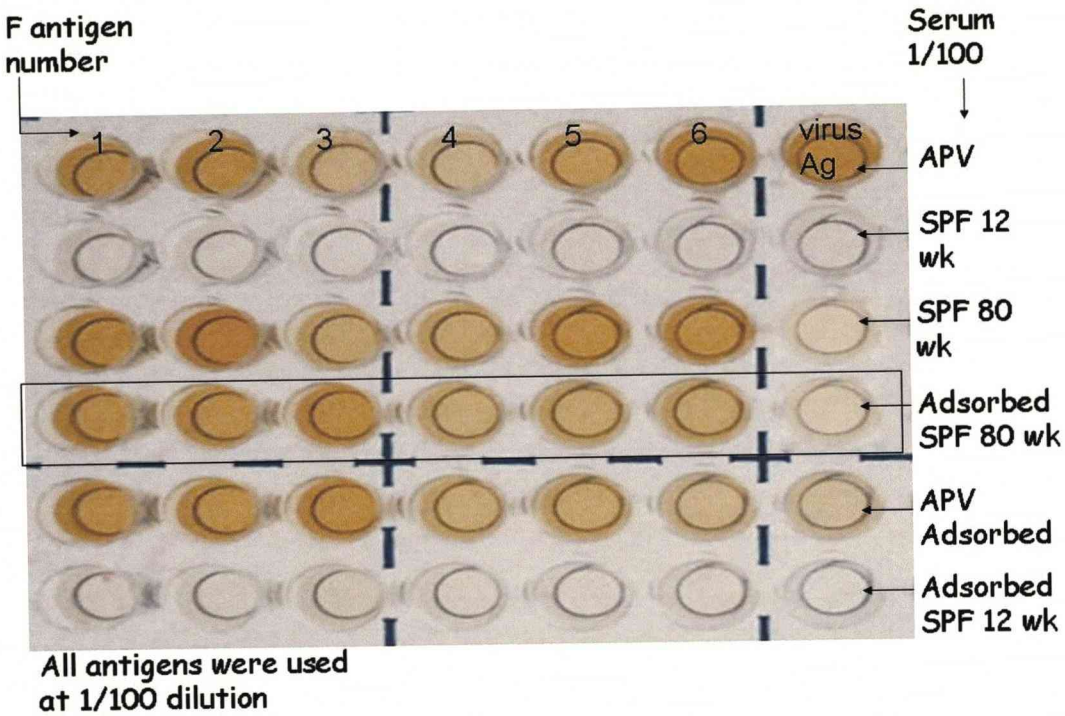


Figure 90. APV ELISA of F antigens and an APV whole virus control (virus Ag). *E.coli* adsorbed SPF 80wk (boxed) serum still gave high background.

Table 24. Absorbencies at 492nm

F section	1	2	3	4	5	6	virus Ag
non adsorbed SPF 80wk	1.4	1.8	0.7	0.7	1.2	1.3	0.2
adsorbed 80wk	1.3	1.7	0.9	0.5	1	1.2	0.3

AGP removed all *E.coli* antibodies from SPF 12 wk and 80wk serum and partially removed them from APV positive serum. However, the comparative ELISA using F antigens and adsorbed and non adsorbed sera showed no improvements to background absorbances (Table 24) with SPF 80wk serum. In addition, high background was observed using the Liverpool developed whole virus antigen. It was considered that APV antibodies might be present in SPF 80wk serum and

therefore an IP staining test was carried out (Figure 91). IP staining suggested that no APV antibodies were present in SPF serum because only APV positive control serum gave a positive reaction (Figure 91).

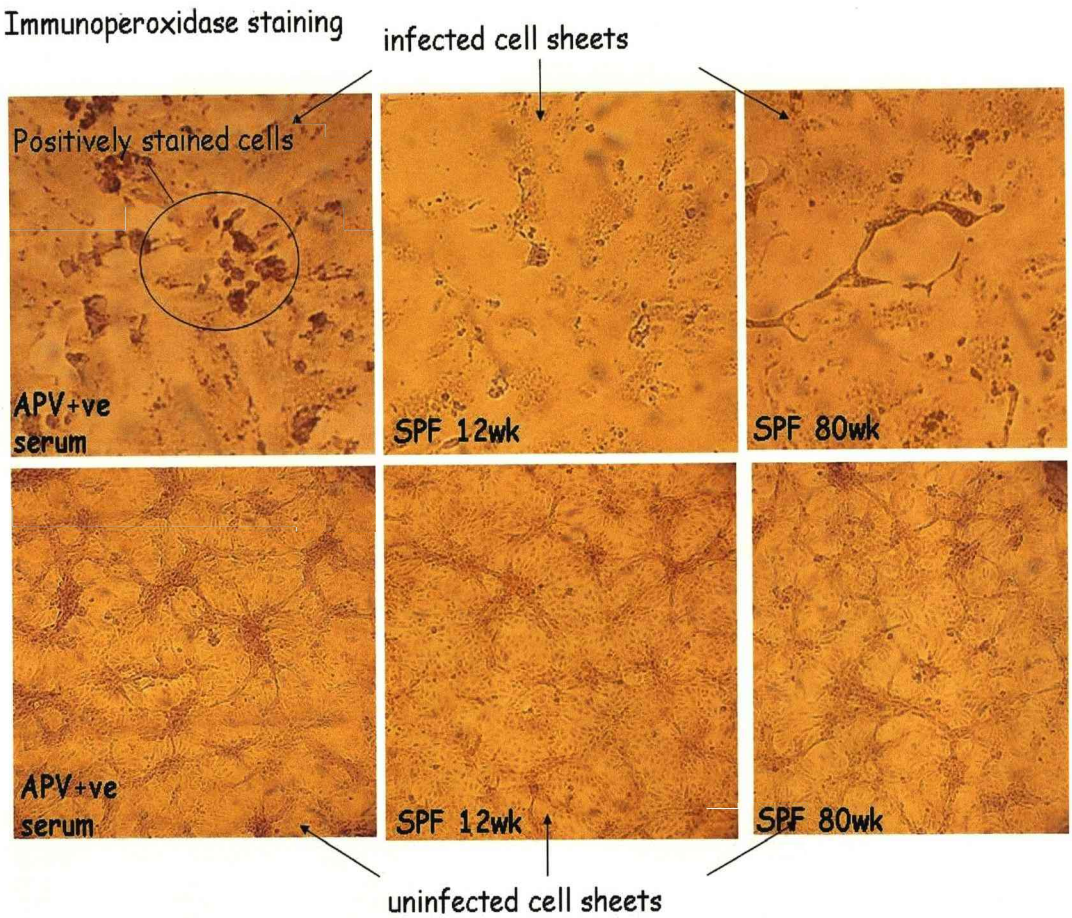


Figure 91. IP staining of uninfected and APV infected vero cell sheets using three different sera (i) APV positive serum (ii) SPF serum fro 12 week old chickens and (iii) SPF serum from 80 week old chickens.

6.9 Results part 4: Virus neutralization

6.9.1 Standard neutralization test

Ninety six-well plates were organized as shown in Table 25. At day 4 pi, CPE (+ Table 25) could be seen in all positive controls and at log_2 13-14 dilutions for subtype A and B antisera. For subtype C antisera CPE was observed at log_2 7-14 dilutions. Therefore, neutralization end points were as follows: (i) log_2 13 for subtype A and B antisera and (ii) log_2 7 for subtype C antisera.

Table 25. Standard virus neutralization test using a ΔSH-G deletion recombinant subtype A APV [72] with subtype A, B and C positive antisera. + indicates CPE

	log_2 dilutions											
Subtype antisera	5	6	7	8	9	10	11	12	13	14	Positive control	Cells only
A	-	-	-	-	-	-	-	-	-	+	+	-
A	-	-	-	-	-	-	-	-	+	+	+	-
B	-	-	-	-	-	-	-	-	+	+	+	-
B	-	-	-	-	-	-	-	-	+	+	+	-
C	-	-	+	+	+	+	+	+	+	+	+	-
C	-	-	+	+	+	+	+	+	+	+	+	-

These end point dilutions provided the basis for the range of dilutions used in subsequent tests (6.8.2) and indicated partial neutralization of a ΔSH-G subtype A APV with subtype C chicken antisera.

6.9.2 Novel neutralization test

Adsorption of the same Subtype A, B and C antiserum (above) with each of the antigenic F sections resulted in a reduction of neutralizing endpoints with subtype A antiserum from log_2 13 (control value) to log_2 10 with F antigen 5 and to log_2 11

with F antigen 4 (Table 26),indicating that neutralizing antibodies in the serum had been themselves neutralized by the those F sections. No change in neutralizing endpoints was observed with F antigens 1, 2, 3 or 6. A reduction in neutralizing endpoints was observed with subtype B anti sera to log_2 12 with antigen 5 and antigen 4 but again no change was observed with F antigens 1, 2, 3 or 6 (Table 27). No reductions in neutralizing endpoints was observed with subtype C anti sera with any F antigen (Table 28)

Table 26. Neutralization of ΔSH-G deletion recombinant subtype A APV with subtype A antisera, following adsorption with subtype A F antigens

	log_2 dilutions							
Fantigen	7	8	9	10	11	12	13	14
1	-	-	-	-	-	-	+	+
1	-	-	-	-	-	-	+	+
1	-	-	-	-	-	+	+	+
1	-	-	-	-	-	-	+	+
2	-	-	-	-	-	-	+	+
2	-	-	-	-	-	-	+	+
2	-	-	-	-	-	-	+	+
2	-	-	-	-	-	-	+	+
3	-	-	-	-	-	-	+	+
3	-	-	-	-	-	-	+	+
3	-	-	-	-	-	-	+	+
3	-	-	-	-	-	-	+	+
4	-	-	-	-	+	+	+	+
4	-	-	-	-	+	+	+	+
4	-	-	-	-	+	+	+	+
4	-	-	-	-	+	+	+	+
5	-	-	-	+	+	+	+	+
5	-	-	-	+	+	+	+	+
5	-	-	-	+	+	+	+	+
5	-	-	-	+	+	+	+	+
6	-	-	-	-	-	-	+	+
6	-	-	-	-	-	-	-	+
6	-	-	-	-	-	-	+	+
6	-	-	-	-	-	-	-	+
control	-	-	-	-	-	-	+	+
control	-	-	-	-	-	-	+	+
control	-	-	-	-	-	-	+	+
control	-	-	-	-	-	-	+	+

Table 27. Neutralization of ΔSH-G deletion recombinant subtype A APV with subtype B antisera, following adsorption with subtype A F antigens

	log_2 dilutions							
Fantigen	7	8	9	10	11	12	13	14
1	-	-	-	-	-	-	+	+
1	-	-	-	-	-	-	+	+
1	-	-	-	-	-	-	-	+
1	-	-	-	-	-	-	+	+
2	-	-	-	-	-	-	+	+
2	-	-	-	-	-	-	+	+
2	-	-	-	-	-	-	-	+
2	-	-	-	-	-	-	+	+
3	-	-	-	-	-	-	+	+
3	-	-	-	-	-	-	+	+
3	-	-	-	-	-	-	+	+
3	-	-	-	-	-	-	+	+
4	-	-	-	-	-	+	+	+
4	-	-	-	-	-	+	+	+
4	-	-	-	-	-	+	+	+
4	-	-	-	-	-	-	+	+
5	-	-	-	-	-	+	+	+
5	-	-	-	-	-	+	+	+
5	-	-	-	-	-	+	+	+
5	-	-	-	-	-	+	+	+
6	-	-	-	-	-	-	+	+
6	-	-	-	-	-	-	+	+
6	-	-	-	-	-	-	+	+
6	-	-	-	-	-	-	+	+
control	-	-	-	-	-	-	+	+
control	-	-	-	-	-	-	+	+
control	-	-	-	-	-	-	+	+
control	-	-	-	-	-	-	+	+

Table 28. Neutralization of ΔSH-G deletion recombinant subtype A APV with subtype C antisera, following adsorption with subtype A F antigens

	log_2 dilutions							
Fantigen	5	6	7	8	9	10	11	12
1	-	-	+	+	+	+	+	+
1	-	-	+	+	+	+	+	+
1	-	-	+	+	+	+	+	+
1	-	-	-	+	+	+	+	+
2	-	-	-	+	+	+	+	+
2	-	-	+	+	+	+	+	+
2	-	-	+	-	+	+	+	+
2	-	-	+	+	+	+	+	+
3	-	-	+	+	+	+	+	+
3	-	-	+	+	+	+	+	+
3	-	-	+	+	+	+	+	+
3	-	-	+	+	+	+	+	+
4	-	-	-	+	+	+	+	+
4	-	-	+	+	+	+	+	+
4	-	-	+	-	+	+	+	+
4	-	-	+	+	+	+	+	+
5	-	-	+	+	+	+	+	+
5	-	-	+	+	+	+	+	+
5	-	-	+	+	+	+	+	+
5	-	-	-	+	+	+	+	+
6	-	-	+	+	+	+	+	+
6	-	-	+	+	+	+	+	+
6	-	-	-	+	+	+	+	+
6	-	-	+	+	+	+	+	+
control	-	-	+	+	+	+	+	+
control	-	-	-	+	+	+	+	+
control	-	-	+	+	+	+	+	+
control	-	-	+	+	+	+	+	+

6.10 Discussion

Six regions of the F protein were successfully amplified, cloned, his tagged and expressed from p-ET30 as recombinant proteins in *E.coli* cells, so justifying the decision to divide it into six smaller sections. Each one yielded detectable levels of protein in his tag ELISAs and Western blots with the exception of F section 2, which was only detected as a weak signal by his tag ELISA. However, only F sections 2, 6 and 5 were detectable by SDS-PAGE, moreover SDS-PAGE suggested that F section 2 was expressed and his tag purified more efficiently than other sections. This posed the question as to how it had it been purified using its his tag but not detected using the his tag monoclonal antibody. It was hypothesisied that following expression, one or more histidines may have been lost. Whilst not interfering with purification, this might have sufficiently altered the tag to leave it unrecognized by the monoclonal antibody. Another suggestion might be that the amino acid combinations in section 2 were able to bind to nickel ions in a similar fashion to a His tag. Although SDS-PAGE analysis demonstrated that F-sections 1, 3 and 4 had been purified at lower levels than 2, 5 and 6, their performances in APV ELISAs were similar. This might indicate that antibodies produced during infection by a natural route preferentially recognized these sections of the F protein. However quantification of each section would be required before such an assertion could be fully justified.

All six antigens tested in ELISAs using subtypes A, B and C APV-positive sera or SPF serum from 12 week old chickens, gave higher absorbances than those using the Liverpool subtype A whole virus as antigen and especially so in ELISAs using subtype C antiserum. Several ELISAs have been developed by others using recombinant subtype C proteins, namely the SH, N and M proteins [2, 3,

59, 63], all of which have been documented as subtype-specific with the exception of a chemically synthesized N protein section of 20 amino acids in length [59]. Alvarez et a.l [59] reported an 85% amino acid homology for this section between all subtypes which probably explained its cross reaction.

Comparison of each F section with equivalent sections in subtype B and C APVs showed amino acid identities to range between 78% and 98% with the exception of sections 2 and 6. These equate to 65% for section 2 with A and B, 46% for section 6 with A and B but only 14% for section 6 with A and C. These percentages made section 6 somewhat of an anomaly with respect to its cross reactivity with C antiserum and suggest the binding may be non-specific However, it was not recognised similarly by monospecific IBV antiserum so it seems likely that this small protein might share common antigenicity with some other region of APV or a host protein upregulated during APV infection.

Relative ELISA absorbancies with subtype B antiserum showed a reduction for antigen 3 from 92% to 61% and from 91 to 60% with antigen 6. This might have been expected for antigen 6 with which it shares only a 46% amino acid identity. However the identity for antigen 3 is 96% and suggests that those small numbers of amino acid changes between A and B subtypes are antigenically important. Relative ELISA absorbances for subtype C antiserum were not calculated because such absorbance readings were so high that they were beyond the linear range and probably also taking the ELISA into a plateau region.

In ELISAs using SPF serum from 80 week old chickens high background was observed with F antigens and to some degree the Liverpool developed whole virus antigen. This was unexpected but it should be borne in mind that it was a test serum maintained by a commercial ELISA company precisely because only highly optimized antigens and purified antigens have proved capable of yielding acceptably low absorbances. Furthermore, the history of such old birds can never be totally known so it seems unnecessary to speculate about these apparently anomalous findings.

Based on the results in this chapter, F antigens 1 - 5 and in particular 4 appear to be useful diagnostic ELISA antigens for detection of antibodies to all APV subtypes in the sera of infected birds (but unresolved concerns with F antigen 6 would eliminate it for recommendation). However the hyperimmune sera tested would have broader specificity than convalescent sera so caution in need in drawing definite conclusions. That said, broader specificity of hyperimmune subtype C antiserum did not result in cross reactivity with subtype A whole virus antigen.

Following adsorption of sera with each F antigen, neutralization tests identified two regions within the F protein that removed that neutralized neutralizing antibodies, F antigen 4 (amino acids 220 – 310 of full length F protein) and F antigen 5 (amino acids 336 – 479 of the full length F protein). These antigens reduced the neutralizing end point dilutions of homologous subtype A antiserum by 2 and 3 $\log_2$ dilutions respectively and by 1 $\log_2$ dilution with subtype B antiserum. No reductions were observed using F antigens 1, 2, 3 and 6. Interestingly, Lounsbach et al. [221] and Werle et al. [188] using F protein

fragments of RSV expressed in *E.coli* and baculovirus respectively demonstrated neutralizing epitopes in amino acids 190 – 289 of the full length RSV F protein, a region that overlaps with the region chosen for APV F antigen 4 here. Other regions of the APV ectodomain did not neutralize and this suggests that the equivalent regions of the F proteins in the two related viruses may continue to share similar functions.

In conclusion, problems expressing hydrophobic membrane proteins in *E.coli* have been documented and it has been suggested that they are most likely due to association of the protein with or incorporation into vital membrane systems [223]. The work described in this chapter promotes a novel approach for addressing these problems. Use of this approach enabled expression and purification of five F antigens that in these studies appeared to be useful in ELISAs for the detection of subtypes A, B and C antibodies. Furthermore F protein fragments enabled the identification of two regions targeted by neutralizing antibodies, one of which is also within an equivalent region identified for RSV F protein [188, 221]. This is the first report identifying regions within subtype A F protein important in the protective immune response.

Chapter 7

General Discussion

This chapter reviews the results of experimental work undertaken in this thesis and gives suggestions for possible future work.

ELISA is the most commonly used serological method for detection of antibodies to viruses, including APV [23, 135, 137, 138, 152, 168, 169]. ELISAs can be performed rapidly, require only tiny volumes of serum and can be automated, enabling many sera to be examined quickly. ELISA is therefore the method of choice for monitoring seroconversion of chickens and turkeys to APV field or vaccine virus. Many commercial and in-house ELISAs have been developed for APV [10, 135-138, 170, 171]. Traditionally, ELISAs using antigens prepared from virus-infected cell cultures. generally work well but have some drawbacks. Antigen preparations generated in this way require virus that can easily be cultured *in vitro* but test specificities can be reduced by the presence of contaminating cellular proteins. Furthermore, Eterradossi et al.. [172] showed that for APV, a subtype A antigen detected subtype A antibodies with greater sensitivity than did a subtype B antigen and vice versa. These problems have been obviated to some extent by expression of recombinant viral proteins [1-7, 63] which allowed individual epitopes to be used and have proven to be sensitive and specific antigens. These can be purified through the use of specifically designed affinity tags placed at the N or C termini of the protein.

In this thesis, three APV subtype A proteins, the nucleocapsid (N), the phosphoprotein (P) and the highly antigenic fusion (F) protein were selected for

expression in an *E.coli* system. They were purified through Ni_2+ capture of their fused his tags. Green fluorescent protein (GFP) was also used throughout the work as a visual reporter of gene expression and subsequent purification.

Many commercial plasmids are available for expressing his tag recombinant proteins in a variety of systems. However, certain APV genes only clone into bacteria with very low efficiency. In particular, using high copy plasmids under typical conditions, resultant purified DNA can be found to contain significant mutations in the APV insert or lack it entirely. In other situations the bacteria are killed by what are assumed to be toxicity effects (C. J. Naylor., D. R. Kapczynski, personal communications). A pUC18 derivative developed by Naylor et al. [72] had been modified to minimise such effects. It was decided to make modifications to this to allow recombinant protein expression in an *E.coli* system. Changes included adding a six his tag stop region to facilitate purification and a T7 termination sequence. This approach was effective and 90% of screened colonies contained desired inserts. Moreover, these cloning strategies were flexible and useful throughout the thesis. Cloning characteristics of p18smahis were comparable with its progenitor plasmid in that it readily accepted APV genes. Nonetheless many resultant clones containing desired inserts had two or more point mutations or deletions, emphasizing the importance of complete sequence analysis.

Expression of GFP from p18smahis was demonstrated, although, expression of N, P and F was more complicated. Expression of F protein was indicated by a 57KDa band (close to the published 59KDa size) [16] by SDS-PAGE and western blot, in a cell lysate of induced *E.coli* containing the F construct. However, purifications yielded no protein. A similar situation occurred when expressing N

and P. It was considered that low levels of expression from p18smahis were responsible and needed improvement. A change of ribosomal binding site sequence and its position relative to the downstream transcribed gene was considered as these were known to affect translation [214, 215]. However, as these changes might have only marginally improved expression and would have been time consuming to implement, a new vector was selected. In addition because GFP can be detected by simple UV analysis, GFP-fusion proteins were made to allow tracking during induction and purification.

Cloning of APV genes and the GFP gene into commercial *E.coli* expression vector p-ET30 was relatively problem-free. However, it is important to note that successful clones were only identified when antibiotic selection could be used to eliminate original template plasmids and select newly developed p-ET30 plasmids from ligation mixtures. Therefore, if clones developed in Chapter 4 (ampicillin resistant) had not been available, p-ET30 (kanamycin resistant) construct development may well have been less efficient.

Expression levels from p-ET30 were significantly improved compared to expression levels from p18smahis. This was best demonstrated by UV microscopy of *E.coli* cells containing the GFP construct. Increasing fluorescence was observed over a three hour induction period and cells appeared structurally sound. Some concern was raised when APV-GFP constructs were analysed in the same way. Although an increase in fluorescent intensity was observed with these constructs, fluorescence was diffused and cells appeared to be losing structural integrity, suggesting problems with APV protein toxicity to *E.coli*. In light of these observations, toxicity could have contributed to the minimal expression of APV proteins experienced during the work documented in Chapter 4.

Purifications of APV-GFP fusion proteins were not successful, although cell lysates were fluorescent. It was considered that concealment of his tag regions within the proteins themselves was responsible for the lack of capture on Ni_2+ charged Ni-NTA matrices, a problem identified when purifying N and P proteins that were not fused with GFP. Those proteins were purified from a native extraction but only after they had been linearised by treatment with 8M urea. This confirmed N terminal his tag concealment in native N and P protein. Unfortunately, although this procedure permitted purification, it rendered them unsuitable as APV ELISA antigen candidates due to destruction of conformational epitopes.. The dual effects of both his tag concealment and protein toxicity led to the consideration that expression of small regions of individual proteins designed to exclude hydrophobic regions could prove less toxic to *E.coli* and reduce concealment problems.

Prior to expression of regions of the APV F0 protein in *E.coli,* a baculovirus expression system was explored. Although *E.coli* expression has the ability to produce antigens rapidly, the main advantage of the baculovirus system is that recombinant proteins expressed in insect cells produce many of the post translational modifications of mammalian cells, including phosphorylation and N-linked and O-linked glycosylation [224] and are therefore capable of producing biologically active proteins. Plaque purified GFP and F2 recombinant baculoviruses were developed and a satisfactory level of GFP expression was demonstrated by UV microscopy. However, F2 protein expression was confirmed only after purification from insoluble material, which made it unsuitable as an ELISA antigen due to the destruction of conformational epitopes. Ultimately this meant that new baculovirus recombinants were required. As discussed above, this meant that lengthy procedures were required. In contrast,

developments in *E.coli* expression would produce greater numbers of test antigens quicker and was therefore favoured.

As already discussed previous attempts to express and purify chosen APV proteins in *E.coli* had indicated problems of toxicity and/or concealment of his tag regions within the proteins being purified. It has been suggested that expression of hydrophobic membrane proteins have toxic effects due to their association with or incorporation into vital membrane systems [223]. A novel approach designed to exclude hydrophobic regions in the APV F0 protein was to express six small regions as individual proteins. Exclusion of hydrophobic regions was designed to eliminate their incorporation into or association with membrane systems and ensuing toxicity effects. Moreover, their reduced size would reduce the chances of his tag concealment. Protein expression and purification improved significantly following these adaptations as each F section was expressed and his tag purified up to three hours post induction as native protein. SDS-PAGE analysis indicated differences in the amount of protein purified for each antigen and showed F antigens 1, 3 and 4 to be at undetectable levels. However, these three antigens were detected by western blot and antigenicity in APV ELISAs were very similar for all F antigens. These results appear to identify F antigens 1, 3 and 4 as regions of the F0 protein with greater immunigenicity than regions 2, 5 and 6, although quantification would be required to accurately determine relative sensitivity.

All six antigens tested in ELISAs using subtypes A, B and C APV-positive sera or SPF serum from 12 week old chickens, gave higher absorbances than those using the Liverpool subtype A whole virus as antigen and especially so in ELISAs using subtype C antiserum. Prior to the work described in this thesis, only one

other antigen has been reported as useful for detection of subtype A, B and C antibodies. This was a chemically synthesized N protein fragment of 20 amino acids in length from subtype C [59]. Alvarez et al. [59] reported an 85% amino acid homology for this section between all subtypes which probably explained its cross reactivity. Comparison of each F section developed here with equivalent sections in subtype B and C APVs showed amino acid identities to range between 84% and 98 % with the exception of sections 2 and 6. These two equated to 65% for section 2 with A and B, 46% for section 6 with A and B but only 14% for section 6 with A and C. These percentages made section 6 somewhat of an anomaly with respect to its cross reactivity with C antiserum and suggested that the binding may be non specific. However, like the other sections it was not recognised by monospecific IBV antiserum so it seems possible that this small protein might share common antigenicity with some other region of APV or a host protein up regulated during APV infection.

Relative ELISA absorbancies with subtype B antiserum showed a reduction for antigen 3 from 92% to 61% and from 91 to 60% with antigen 6. This might have been expected for antigen 6 with which it shares only a 46% amino acid identity. However the identity for antigen 3 is 96% and suggests that those small numbers of amino acid changes between A and B subtypes are antigenically important. Relative ELISA absorbances for subtype C antiserum were not calculated because such absorbance readings were so high that they were beyond the linear range and probably also taking the ELISA into a plateau region.

Commercial testing of F antigens in APV ELISAs initially produced desired results however when tested against SPF serum from 80week old chickens high

background was observed. Testing of the serum in Liverpool produced the similar high absorbencies with F antigens and to some degree with the developed whole virus antigen. Although this was unexpected, it is important to note that it was a commercial test serum maintained precisely because only highly optimized antigens have proved capable of yielding acceptably low absorbances. Furthermore, the nature of sera from birds of such an age can never be totally known so it seems unnecessary to speculate about these apparently anomalous findings. Hence, F antigens 1 - 5 and in particular 4 appear to be promising diagnostic ELISA antigens for detection of antibodies to all APV subtypes in the sera of infected birds but unresolved concerns with F antigen 6 would eliminate it for recommendation. The hyperimmune sera tested are likely to have broader specificity than convalescent sera so caution is needed in drawing definite conclusions. That said, broader specificity of hyperimmune subtype C antiserum did not result in cross reactivity with subtype A whole virus antigen. It would be useful therefore to test F antigens against both hyperimmune and convalescent antisera of all subtypes to address these issues. Quantification of F antigens would also benefit the accurate determination of relative sensitivities.

When neutralization tests were conducted in cell culture following adsorption of sera with each F antigen, two regions were identified within the F protein that neutralized the activity of the antibodies, F antigen 4 (amino acids 220 – 310 of full length F protein) and F antigen 5 (amino acids 336 – 479 of the full length F protein). These antigens reduced the neutralizing end point dilutions of homologous subtype A antiserum by 2 and 3 $\log_2$ dilutions respectively and by 1 $\log_2$ dilution with subtype B antiserum. No reductions were observed using F antigens 1, 2, 3 or 6. Interestingly, Lounsbach et al. [221] and Werle et al. [188]

using F protein fragments of RSV expressed in *E.coli* and baculovirus respectively demonstrated neutralizing epitopes in amino acids 190 – 289 of the full length RSV F protein, a region that overlaps with the region chosen for APV F antigen 4 here. Other regions of the APV ectodomain did not neutralize and this suggests that the equivalent regions of the F proteins in the 2 related viruses may continue to share similar functions.

Significant progress was made throughout this work to express and his tag purify APV subtype A proteins for use as ELISA antigens. During development indications of APV protein toxicity to E.coli, as early as 1 hour post induction and N and C terminal concealment of his tag regions were demonstrated. In contrast, APV subtype C recombinant N protein was successfully purified 4 hours post induction and with no report of concealment of N-terminal his tag regions when expressed in and purified from the same strain of *E.coli* cells by Gulati et al.. [3]. This demonstrates considerable differences between subtype A and C nucleocapsid proteins when expressed in *E.coli,* which might be partly explained by the relatively low amino acid identity of 69% [55] between the US subtype C and the European subtype A and B nucleocapsid proteins.

Problems expressing and purifying subtype A proteins were overcome by novel expression of regions of the full length F protein. F antigens 1–5 in these studies appeared to be useful in ELISAs for the detection of subtypes A, B and C antibodies, a finding that agrees with work of Tarpy et al. [176] who inadvertently identified the fusion protein as a potential antigen candidate for such purposes. Only one other universal APV ELISA antigen has previously been reported [59].

In addition to their use in ELISAs, F protein fragments enabled the identification of two regions targeted by neutralizing antibodies, one of which is within an equivalent region identified for the RSV F protein [188, 221] suggesting that the equivalent regions of the F proteins in the two related viruses share similar functions. This is the first report identifying regions within the subtype A F protein likely to be important in the protective immune response and it is reasonable to infer that these same regions may stimulate or enhance a protective immune response, either in their present form or after further narrowing down to constituent epitopes. Shorter F sections from within these regions could be used in identical tests with a view to identifying epitopes recognized by the neutralizing antibodies. These would be conveniently made by chemical synthesis and this technique would further allow convenient modification of individual amino acids to show their individual importance.

The importance of these epitopes might be further extended by introducing mutated epitopes into the ΔSH-G deletion virus using our reverse genetics system. If the mutation did not prevent virus rescue, absence of neutralization would be further evidence of the importance of the epitope. In addition it would be interesting to compare the protective capacity of such a virus with one not containing epitope modification.

Incorporation of F sections 4 and 5 as additions to the full length genome of recombinant APV vaccine candidates is also a possibility. Such rescued recombinants might have the potential to increase the stimulation of neutralizing antibody production due to the presence of increased F epitopes involved in virus neutralization. It is likely that the F section(s) would be positioned between M2

and G genes because this has proved to produce acceptable levels when adding the GFP gene.

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Appendix

dNTP solution

- 20µl dATP (100mM)
- 20µl dCTP (100mM)
- 20µl dGTP (100mM)
- 20µl dTTP (100mM)
- 120µl double processed tissue culture water (Sigma W3500)

Miniprep TENS buffer

- 0.37g EDTA
- 5g SDS (Sigma L3771)
- NaOH
- 0.88g Tris HCL (sigma T-3253)
- 0.53g Tris base (sigma T-6066)

Agarose gel electrophoresis 1.4% (Agarose and buffer was adjusted accordingly to achieve a desired %) NB 10x TBE buffer was diluted 10 fold with distilled water for use. Gels were prepared in a conical flask.

- 0.5g Agarose powder (Promega v-3121)
- 35ml 1x TBE buffer (Invitrogen 10x TBE 15581-044)
- Swirl mix then heat for 40 seconds microwave power setting 100%
- Swirl cool under cold tap
- Pour into gel tank
- Allow the gel to set for ≈ 15 mins then overlay with TBE buffer until buffer level is just below the surface of the tank

Molecular weight standards.

- 50µl Hind λ (invitrogen, UK)
- 25µl φ x174 (invitrogen UK)
- 25µl DNA load buffer (see below)
- 2µl / run was used

DNA load buffer

- 0.25% Bromophenol blue (sigma B-0126)
- 30% Ficoll type 400 (Sigma catalogue number F4375)
- In a total volume of 10ml distilled water.

Ethidium bromide (sigma E-1510)

- 0.05% in 250ml distilled water

Exo / Sap

- 15 microlitres PCR product
- 1 microlitre SAP
- 0.15 microlitres Exo 1
- PCR Block 37°C for 30 minutes, 80°C for 10 minutes, hold at 4°C.

Exo

- 0.5µl / 50µl PCR product
- PCR Block 37°C for 30 minutes, 80°C for 10 minutes, hold at 4°C.

SDS-PAGE recipes

Separating gels 10%

- 12.5ml of Acrylamide stock solution (40%) 37:1 (sigma #A6050)
- 0.5 ml of 10% SDS (Sigma L3771) in distilled water.
- 6.25ml of separating gel buffer (see below)
- 30.5ml of distilled water
- 25 µl of TEMED (sigma T-7024)
- 250µl of 10% APS made fresh each time (sigma L3771)

Stacking gels 5%

- 2.5 ml of Acrylamide stock solution (40%) 37:1
- 0.2 ml of 10% SDS in distilled water.
- 5 ml of stacking gel buffer (see below)
- 12.3 ml of distilled water
- 20 µl of TEMED

- 100µl of 10% APS

Ammonium persulphate 10%

- 0.1g APS made up to 1ml in distilled water.

Electrode running buffer (concentrate)

- 30.3 g Tris base (sigma T-6066)
- g Glycine (sigma G-8898)
- Dissolved in distilled water and made up to 1ltr (store at -4^0c keep for 1 month.
- For use, 200ml of concentrate plus 20ml of 10% SDS, make up to 2ltr.

Stacking gel buffer 0.5M pH 6.8

- 0.283g Tris base (sigma T-6066)
- 7.51 g Tris HCL (sigma T-3253)
- Dissolved in distilled water and made up to 100ml (store at -4^0c keep for 1 month.

Separating gel buffer 3M pH 8.8

- 30.21g Tris base (sigma T-6066)
- 7.96 g Tris HCL (sigma T-3253)
- Dissolved in distilled water and made up to 100ml (store at -4^0c keep for 1 month.

Protein Sample Buffers

None reducing

- 10ml Stacking gel buffer
- 0.8g SDS
- 4ml Glycerol (sigma G-8773)
- 0.4mg bromophenol blue (sigma B-0126)
- Made up to 20ml with distilled water. Store room temperature.

Reducing

Same as above but add 1ml 2ME (sigma M-3148)

Gel staining

Gels were stained overnight and de-stained as desired.

Stain

- 1g Coomassie blue (BDH G-250)
- 250ml Methanol
- 35ml Acetic acid (glacial)
- 215ml distilled water
- Filter and store at room temp

De-stain

- 100ml Methanol
- 35ml Acetic acid (glacial)
- 365ml distilled water
- Store at room temp

Gel preparation

- Separating gels were prepared in a buchner flask with the exception of the 10% APS solution
- Solution was de-gassed under reduced pressure for at least 10 mins. This reduced the risk of oxygen inhibition of acrylamide polymerisation.
- 10% APS was added to the de-gassed solution and then poured immediately between the glass plates.
- Gel solution was gently overlaid with water using a syringe.
- Gel was allowed to set.
- Stacking gel was prepared in the same way.
- Water was removed from the separating gel and a 24 well comb partially insert.
- The stacking gel was poured and the comb completely inserted.
- Gel was allowed to set.
- Comb was removed.
- Electrode running buffer applied to both sections of the gel apparatus.

Protein Sample preparation/loading

- Samples were mixed 50:50 with sample buffer, 50µl total volume.
- These were boiled for 5mins.
- Allowed to cool
- Centrifuged at 13.2 x 1000rpm for 30 seconds
- 40µl was loaded into the formed wells.
- Gels were run at either 75 mAmps (one gel) or 145mAmps (two gels) for 1hr 10mins

Molecular weight markers

Colour burst markers (Sigma C-4105) needed no preparation prior to loading. 10µl was used per run.

Wide molecular weight markers (Sigma M-4038) were prepared and used according to Sigma recommended protocols

PBS 10x stock solution

- 80g sodium chloride (BDH 10241)
- 2g potassium chloride (BDH 10198)
- 11.5g $N_{a2}HPO_4$ (BDH 10249)
- 2g KH_2PO_4 (BDH 10203)
- Made up to 1 litre with distilled water

DAB substrate buffer (0.1M phosphate pH 6.0)

- 2.12g $N_{a2}HPO_4$ $12H_2O$
- 14.67g NaH_2PO_4 $2H_2O$
- 0.5ml $Tween_{80}$
- Made up to 1 litre with distilled water

DAB solution

- One DAB tablet (Sigma, D5905)
- 15ml of DAB substrate buffer

- 12µl 30% H_2O_2 (H-1009)

Electrode transfer buffers

	Anode 1	Anode 2	Cathode
• pH	10.4	10.4	7.6
• Tris base (Sigma T8524)	18.5	1.51g	
• 6 amino hexonoic acid (Sigma A2504)			2.6g
• Methanol	100ml		100ml

100ml

- All made to a final volume of 500ml with distilled water

ELISAs

CBC Buffer

	500ml	100ml
•	0.75g Na_2CO_3	0.15g
•	1.47g $NaHCO_3$	0.29g

Make up to volume with distilled water. Store for 1 week at 0-4^{0}C

Wash Solution – ELISA's

	2.5Ltr	1Ltr
•	292g N_aCL	116.8g
•	5.35g $N_{a2}HPO_4$	2.14g
•	1.95g NaH_2PO_4 ($2H_2O$)	0.78g
•	Tween 80 2.5ml	1ml

Tween 80 to be added last just before storing. Dilute 1 in 2 prior to use.

Orthophenylene diamine (OPD) Substrate

0.2M Disodium hydrogen phosphate (Na_2HPO_4) anhydrous

- 28.4g Na_2HPO_4 / Litre H_20

0.1M Citric Acid

- 21.01g Citric acid ($C_6H_8O7.H_20$) / Litre

0.2M disodium hydrogen phosphate ~200ml added to 0.1M citric acid (243ml) with stirring until the pH reaches 5.0.
Made up to 1 litre with deionised water.
0.4g of OPD, (20 tablets of 20mg – Sigma p5412) added, mixed well in the dark and aliquoted into 12 or 24 ml amounts.
Stored at -20°C till required. Prior to use it is bought to room temperature (in the dark) and 20µl of 30% H_2O_2 (Sigma, UK H-1009) is added per 24ml.

2.5 M H_2SO_4

- 245g/Litre distilled water or 134ml/Litre distilled water

Gram staining

- Place 0.5µl of lysate, mixed with an equal volume of distilled water onto a microscope slide, air dry and fix over a Bunsen flame.
- Flood Heat-fixed smear with crystal violet and leave for 1 minute
- Rinse with tap water
- Flood Heat-fixed smear with grams iodine and leave for 1 minute
- Rinse briefly with tap water
- Add acetone for 2-3 seconds
- Rinse immediately with tap water
- Add safranin for 30 seconds
- Rinse and blot dry
- Apply small drop of immersion oil and examine using an oil-immersion objective under light microscopy.

Tissue culture medium

- 500ml Dulbecco's modified eagles medium (sigma D6546)
- 5ml 200mM L-Glutamine (sigma G7513)
- 2ml Pen and Strep solution (see pen/strep below)
- 1ml Fungizone (Invitrogen 15290-026)

Tissue culture medium + serum

- Same as above plus 5% foetal bovine serum

Pen/Strep

- 600mg crystapen (Britannia pharmaceuticals ltd)
- 1g streptomycin (sigma S-9137)
- Made up in 40ml of tissue culture water (sigma W3500)

Ni-NTA elution buffer

- 0.61g Na_2HPO_4 anhydrous
- 0.11g NaH_2PO_4 ($2H_2O$)
- 1.75g NaCl
- 2.1g imidazole (sigma I0250)
- Made up in 100ml of tissue culture water (sigma W3500)